RITUXAN™
Rituximab

DESCRIPTION

The RITUXAN (Rituximab) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. The antibody is an IgG_1 kappa immunoglobulin containing murine light- and heavy-chain variable region sequences and human constant region sequences. Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids (based on cDNA analysis) and has an approximate molecular weight of 145 kD. Rituximab has a binding affinity for the CD20 antigen of approximately 8.0 nM.

The chimeric anti-CD20 antibody is produced by mammalian cell (Chinese Hamster ovary) suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product. The anti-CD20 antibody is purified by affinity and ion exchange chromatography. The purification process includes specific viral inactivation and removal procedures.

RITUXAN is a sterile, clear, colorless, preservative-free liquid concentrate for intravenous (IV) administration. RITUXAN is supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials. The product is formulated for intravenous administration in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, and Sterile Water for Injection. The pH is adjusted to 6.5.

CLINICAL PHARMACOLOGY

General

Rituximab binds specifically to the antigen CD20 (human B-lymphocyte-restricted differentiation antigen, Bp35), a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes.[1,2] The antigen is also expressed on >90% of B-cell non-Hodgkin's lymphomas (NHL)[3] but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues.[4] CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation,[4] and possibly functions as a calcium ion channel.[5] CD20 is not shed from the cell surface and does not internalize upon antibody binding.[6] Free CD20 antigen is not found in the circulation.[2]

Pre-clinical Pharmacology and Toxicology

Mechanism of Action: The Fab domain of Rituximab binds to the CD20 antigen on B-lymphocytes and the Fc domain recruits immune effector functions to mediate B-cell lysis *in vitro*. Possible mechanisms of cell lysis include complement-dependent cytotoxicity (CDC)[7] and antibody-dependent cellular cytotoxicity (ADCC). The antibody has been shown to induce apoptosis in the DHL-4 human B-cell lymphoma line.[8]

Normal Tissue Cross-reactivity: Rituximab binding was observed on lymphoid cells in the thymus, the white pulp of the spleen, and a majority of B-lymphocytes in peripheral blood and lymph nodes. Little or no binding was observed in non-lymphoid tissues examined.

Human Pharmacokinetics/Pharmacodynamics

In patients given single doses at 10, 50, 100, 250 or 500 mg/m² as an IV infusion, serum levels and the half-life of Rituximab were proportional to dose. In 9 patients given 375 mg/m² as an IV infusion for four doses, the mean serum half-life was 59.8 hours (range 11.1 to 104.6 hours) after the first infusion and 174 hours (range 26 to 442 hours) after the fourth infusion. The wide range of half-lives may reflect the variable tumor burden among patients and the changes in CD20 positive (normal and malignant) B-cell populations upon repeated administrations.

Rituximab at a dose of 375 mg/m² was administered as an IV infusion at weekly intervals for four doses to 166 patients. The peak and trough serum levels of Rituximab were inversely correlated with baseline values for the number of circulating CD20 positive B cells and measures of disease burden. Median steady-state serum levels were higher for responders compared to nonresponders; however, no difference was found in the rate of elimination as measured by serum half-life. Serum levels were higher in patients with International Working Formulation (IWF) subtypes B, C, and D as compared to those with subtype A. Rituximab was detectable in the serum of patients three to six months after completion of treatment.

The pharmacokinetic profile of Rituximab when administered as six infusions of 375 mg/m² in combination with six cycles of CHOP chemotherapy was similar to that seen with Rituximab alone.

Administration of RITUXAN resulted in a rapid and sustained depletion of circulating and tissue-based B cells. Lymph node biopsies performed 14 days after therapy showed a decrease in the percentage of B-cells in seven of eight patients who had received single doses of Rituximab ≥100 mg/m². [9] Among the 166 patients in the pivotal study, circulating B-cells (measured as CD19+ cells) were depleted within the first three doses with sustained depletion for up to 6 to 9 months post-treatment in 83% of patients. One of the responding patients (1%), failed to show significant depletion of CD19+ cells after the third infusion of Rituximab as compared to 19% of the nonresponding patients. B-cell recovery began at approximately six months following completion of treatment. Median B-cell levels returned to normal by twelve months following completion of treatment.

There were sustained and statistically significant reductions in both IgM and IgG serum levels observed from 5 through 11 months following Rituximab administration. However, only 14% of patients had reductions in IgG and/or IgM serum levels, resulting in values below the normal range.

CLINICAL STUDIES

A multicenter, open-label, single-arm study was conducted in 166 patients with relapsed or refractory low-grade or follicular B-cell NHL who received 375 mg/m² of RITUXAN given as an IV infusion weekly for four doses. Patients with tumor masses >10 cm or with >5,000 lymphocytes/μL in the peripheral blood were excluded from the study. The overall response rate (ORR) was 48% (80/166) with a 6% (10/166) complete response (CR) and a 42% (70/166) partial response (PR) rate. Disease-related signs and symptoms (including B-symptoms) were present in 23% (39/166) of patients at study entry and resolved in 64% (25/39) of those patients. The median time to onset of response was 50 days and the median duration of response is projected to be 10 to 12 months.

In a multivariate analysis, the ORR was higher in patients with IWF B, C, and D histologic subtypes as compared to IWF A subtype (58% vs. 12%), higher in patients whose largest lesion was <5 cm vs. >7 cm in greatest diameter (55% vs. 38%), and higher in patients with chemosensitive relapse as compared to chemoresistant (defined as duration of response <3 months) relapse (53% vs. 36%). ORR in patients previously treated with autologous bone marrow transplant was 78% (18/23). The following factors were not associated with a lower response rate: age ≥ 60 years, extranodal disease, prior anthracycline therapy, and bone marrow involvement.

In a second multicenter, multiple-dose study, 37 patients with relapsed or refractory B-cell NHL received 375 mg/m² of RITUXAN as an IV infusion once weekly for four doses.[10,11] The ORR was 46% with a median duration of response of 8.6 months (range 2.6 to 26.2+). Single doses of up to 500 mg/m² were well-tolerated.[9]

Twenty patients have received two courses and one patient has received three courses of RITUXAN as 4 weekly infusions of 375 mg/m² per infusion. The percentage of patients reporting adverse events upon retreatment was similar to that reported following the first course, although the incidence of specific adverse events differed (see ADVERSE EVENTS). All patients had obtained an objective clinical response (CR or PR) to the first course of RITUXAN™ (Rituximab); upon retreatment, 6 of 12 patients evaluable for response obtained a complete or partial remission.

Twenty-nine patients with relapsed or refractory, bulky (single lesion of >10 cm in diameter), low grade NHL received 375 mg/m² of RITUXAN as four weekly infusions. The overall incidence of adverse events and the incidence of Grade 3 and 4 adverse events was higher in patients with bulky disease than in patients with non-bulky disease (see ADVERSE EVENTS). Ten of 21 patients evaluable for response have obtained a complete or partial remission.

INDICATIONS AND USAGE

RITUXAN is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma.

CONTRAINDICATIONS

RITUXAN is contraindicated in patients with known Type I hypersensitivity or anaphylactic reactions to murine proteins or to any component of this product. (See WARNINGS.)

WARNINGS

RITUXAN is associated with hypersensitivity reactions which may respond to adjustments in the infusion rate. Hypotension, bronchospasm, and angioedema have occurred in association with RITUXAN infusion as part of an infusion-related symptom complex. RITUXAN infusion should be interrupted for severe reactions and can be resumed at a 50% reduction in rate (e.g., from 100 mg/hr to 50 mg/hr) when symptoms have completely resolved. Treatment of these symptoms with diphenhydramine and acetaminophen is recommended; additional treatment with bronchodilators or IV saline may be indicated. In most cases, patients who have experienced non-life-threatening reactions have been able to complete the full course of therapy. (See DOSAGE and ADMINISTRATION.) Medications for the treatment of hypersensitivity reactions, e.g., epinephrine, antihistamines and corticosteroids should be available for immediate use in the event of a reaction during administration.

Infusions should be discontinued in the event of serious or life-threatening cardiac arrhythmias. Patients who develop clinically significant arrhythmias should undergo cardiac monitoring during and after subsequent infusions of RITUXAN. Patients with preexisting cardiac conditions including arrhythmias and angina have had recurrences of these events during RITUXAN therapy and should be monitored throughout the infusion and immediate post-infusion period.

PRECAUTIONS

Laboratory Monitoring: Complete blood counts (CBC) and platelet counts should be obtained at regular intervals during RITUXAN therapy and more frequently in patients who develop cytopenias (see ADVERSE EVENTS).

Drug/Laboratory Interactions: There have been no formal drug interaction studies performed with RITUXAN.

HAMA/HACA Formation: Human anti-murine antibody (HAMA) was not detected in 67 patients evaluated. Less than 1.0% (3/355) of patients evaluated for human anti-chimeric antibody (HACA) were positive. Patients who develop HAMA/HACA titers may have allergic or hypersensitivity reactions when treated with this or other murine or chimeric monoclonal antibodies.

Immunization: The safety of immunization with any vaccine, particularly live viral vaccines, following RITUXAN therapy has not been studied. The ability to generate a primary or anamnestic humoral response to any vaccine has also not been studied.

Carcinogenesis, Mutagenesis, Impairment of Fertility: No long-term animal studies have been performed to establish the carcinogenic or mutagenic potential of RITUXAN, or to determine its effects on fertility in males or females. Individuals of childbearing potential should use effective contraceptive methods during treatment and for up to 12 months following RITUXAN therapy.

Pregnancy Category C: Animal reproduction studies have not been conducted with RITUXAN. It is not known whether RITUXAN can cause fetal harm when administered to a pregnant woman or whether it can affect reproductive capacity. Human IgG is known to pass the placental barrier, and thus may potentially cause fetal B-cell depletion; therefore, RITUXAN should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether RITUXAN is excreted in human milk. Because human IgG is excreted in human milk and the potential for absorption and immunosuppression in the infant is unknown, women should be advised to discontinue nursing until circulating drug levels are no longer detectable. (See CLINICAL PHARMACOLOGY.)

Pediatric Use: The safety and effectiveness of RITUXAN in children have not been established.

ADVERSE REACTIONS

Safety data are based on 315 patients treated in five single-agent studies of RITUXAN. This includes patients with bulky disease (lesions >10 cm), those who have received more than one course of RITUXAN, and patients receiving 375 mg/m² for eight doses.

Infusion-Related Events: An infusion-related symptom complex consisting of fever and chills/rigors occurred in the majority of patients during the first RITUXAN infusion. Other frequent infusion-related symptoms included nausea, urticaria, fatigue, headache, pruritus, bronchospasm, dyspnea, sensation of tongue or throat swelling (angioedema), rhinitis, vomiting, hypotension, flushing, and pain at disease sites. These reactions generally occurred within 30 minutes to 2 hours of beginning the first infusion, and resolved with slowing or interruption of the RITUXAN infusion and with supportive care (IV saline, diphenhydramine, and acetaminophen). The incidence of infusion-related events decreased from 80% (7% Grade 3/4) during the first infusion to approximately 40% (5% to 10% Grade 3/4) with subsequent infusions. Mild to moderate hypotension requiring interruption of RITUXAN infusion with or without the administration of IV saline occurred in 32 (10%) patients. Isolated occurrences of severe reactions requiring epinephrine have been reported in patients receiving RITUXAN for other indications. Angioedema was reported in 41 (13%) patients and was serious in one patient. Bronchospasm occurred in 25 (8%) patients; one-quarter of these patients were treated with bronchodilators. A single report of bronchiolitis obliterans was noted.

Immunologic Events: RITUXAN induced B-cell depletion in 70 to 80% of patients and was associated with decreased serum immunoglobulins in a minority of patients. The incidence of infection does not appear to be increased. During the treatment period, 50 patients in the pivotal trial developed 68 infectious events; 6 (9%) were Grade 3 in severity and none were Grade 4 events. Of the

Samsung Bioepis Exhibit No. 1002
Page 7538

6 serious infectious events, none were associated with neutropenia. The serious bacterial events included sepsis due to Listeria (n=1), Staphylococcal bacteremia (n=1) and polymicrobial sepsis (n=1). In the post-treatment period (30 days to 11 months following the last dose), bacterial infections included sepsis (n=1); significant viral infections included herpes simplex infections (n=2) and herpes zoster (n=3).

Retreatment Events: Twenty-one patients have received more than one course of RITUXAN™ (Rituximab). The percentage of patients reporting any adverse event upon retreatment was similar to the percentage of patients reporting adverse events upon initial exposure. The following adverse events were reported more frequently in retreated subjects: asthenia, throat irritation, flushing, tachycardia, anorexia, leukopenia, thrombocytopenia, anemia, peripheral edema, dizziness, depression, respiratory symptoms, night sweats, and pruritus.

Hematologic Events: During the treatment period (up to 30 days following last dose) severe thrombocytopenia occurred in 1.3% of patients, severe neutropenia occurred in 1.9% of patients, and severe anemia occurred in 1.0% of patients. A single occurrence of transient aplastic anemia (pure red cell aplasia) and two occurrences of hemolytic anemia following RITUXAN therapy were reported.

Cardiac Events: Four patients developed arrhythmias during RITUXAN infusion. One of the four discontinued treatment because of ventricular tachycardia and supraventricular tachycardias. The other three patients experienced trigeminy (1) and irregular pulse (2) and did not require discontinuation of therapy. Angina was reported during infusion and myocardial infarction occurred 4 days post-infusion in one subject with a prior history of myocardial infarction.

Table 1.

Adverse Events ≥5% of Patients (N=315)

	Incidence All Grades	
	N	%
Any Adverse Event	**275**	**87**
Body As A Whole		
Fever	154	49
Chills	102	32
Asthenia	49	16
Headache	43	14
Throat Irritation	19	6
Abdominal Pain	18	6
Cardiovascular System		
Hypotension	32	10
Digestive System		
Nausea	55	18
Vomiting	23	7
Hemic and Lymphatic System		
Leukopenia	33	11
Thrombocytopenia	25	8
Neutropenia	21	7
Metabolic and Nutritional System		
Angioedema	41	13
Musculo-Skeletal System		
Myalgia	21	7
Nervous System		
Dizziness	23	7
Respiratory System		
Rhinitis	25	8
Bronchospasm	24	8
Skin and Appendages		
Pruritus	32	10
Rash	31	10
Urticaria	24	8

Severe and life-threatening (Grade 3 and 4) events were reported in 10% (32/315) of patients. The following Grade 3 and 4 adverse events were reported: neutropenia (1.9%), chills (1.6%), leukopenia and thrombocytopenia (1.3% for each), hypotension, anemia, bronchospasm, and urticaria (1.0% for each), headache, abdominal pain, arrhythmia (0.6% for each), and asthenia, hypertension, nausea, vomiting, coagulation disorder, angioedema, arthralgia, pain, rhinitis, increased cough, dyspnea, bronchiolitis obliterans, hypoxia, asthma, pruritus, and rash (one patient each, 0.3%).

The following adverse events occurred in ≥1.0% but <5.0% of patients, in order of decreasing incidence: flushing, artbralgia, diarrhea, anemia, cough increase, hypertension, lacrimation disorder, pain, hyperglycemia, back pain, peripheral edema, paresthesia, dyspepsia, chest pain, anorexia, anxiety, malaise, tachycardia, agitation, insomnia, sinusitis, conjunctivitis, abdominal enlargement, postural hypotension, LDH increase, hypocalcemia, hypesthesia, respiratory disorder, tumor pain, pain at injection site, bradycardia, hypertonia, nervousness, bronchitis, and taste perversion.

The proportion of patients reporting any adverse event was similar in patients with bulky disease and those with lesions <10 cm in diameter. However, the incidence of dizziness, neutropenia, thrombocytopenia, myalgia, anemia and chest pain was higher in patients with lesions >10 cm. The incidence of any Grade 3 and 4 event was higher (31% vs. 13%) and the incidence of Grade 3 or 4 neutropenia, anemia, hypotension, and dyspnea was also higher in patients with bulky disease compared with patients with lesions <10 cm.

OVERDOSAGE

There has been no experience with overdosage in human clinical trials. Single doses higher than 500 mg/m² have not been tested.

DOSAGE AND ADMINISTRATION

Usual Dose:

The recommended dosage of RITUXAN is 375 mg/m² given as an IV infusion once weekly for four doses (days 1, 8, 15, and 22). RITUXAN may be administered in an outpatient setting. **DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. (See Administration.)**

Instructions for Administration

Preparation for Administration: Use appropriate aseptic technique. Withdraw the necessary amount of RITUXAN and dilute to a final concentration of 1 to 4 mg/mL into an infusion bag containing either 0.9% Sodium Chloride USP or 5% Dextrose in Water USP. Gently invert the bag to mix the solution. Discard any unused portion left in the vial. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration.

RITUXAN solutions for infusion are stable at 2° to 8°C (36° to 46°F) for 24 hours and at room temperature for an additional 12 hours. No incompatibilities between RITUXAN and polyvinylchloride or polyethylene bags have been observed.

Administration: DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. Hypersensitivity reactions may occur (see WARNINGS). Premedication, consisting of acetaminophen and diphenhydramine, should be considered before each infusion of RITUXAN™ (Rituximab). Premedication may attenuate infusion-related events. Since transient hypotension may occur during RITUXAN infusion, consideration should be given to withholding antihypertensive medications 12 hours prior to RITUXAN infusion.

First Infusion: The RITUXAN solution for infusion should be administered intravenously at an initial rate of 50 mg/hr. RITUXAN should not be mixed or diluted with other drugs. If hypersensitivity or infusion-related events do not occur, escalate the infusion rate in 50 mg/hr increments every 30 minutes, to a maximum of 400 mg/hr. If hypersensitivity or an infusion-related event develops, the infusion should be temporarily slowed or interrupted (see WARNINGS). The infusion can continue at one-half the previous rate upon improvement of patient symptoms.

Subsequent Infusions: Subsequent RITUXAN infusions can be administered at an initial rate of 100 mg/hr, and increased by 100 mg/hr increments at 30-minute intervals, to a maximum of 400 mg/hr as tolerated.

Stability and Storage: RITUXAN vials are stable at 2° to 8°C (36° to 46°F). Do not use beyond expiration date stamped on carton. RITUXAN vials should be protected from direct sunlight.

HOW SUPPLIED

RITUXAN is supplied as 100 mg and 500 mg of sterile, preservative-free, single-use vials.
Single unit 100 mg carton: Contains one 10 mL vial of RITUXAN (10 mg/mL).
NDC 50242-051-21
Single unit 500 mg carton: Contains one 50 mL vial of RITUXAN (10 mg/mL).
NDC 50242-053-06

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Samsung Bioepis Exhibit No. 1002
Page 7539

Consolidated List of Documents cited in opposition to EP2359834 B1				
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Samsung Bioepis Exhibit No. 1002
Page 7540

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Commercial monoclonal antibodies 2006 (all modes of administration)	D31			Annex I
Commercial monoclonal antibodies 2006 (I.V. administration)	D32			Annex II
Declaration of Dr Miano	D33	D33		
Rouet *et al.*, (2014) *FEBS Letters.* 588: 269-277	D34	D34		
Summary of antibodies listed in D20/D21 Tables	D35	D35		
Graphical representation of D20 Table 2	D36	D36		
Summary of antibody products approved by EMA since filing date	D37	D37		
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Samsung Bioepis Exhibit No. 1002
Page 7541

AUXILIARY REQUEST 2

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein the composition is administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration.

Samsung Bioepis Exhibit No. 1002
Page 7542

AUXILIARY REQUEST 1

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein administration is by intravenous infusion.

Samsung Bioepis Exhibit No. 1002
Page 7543

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Acknowledgement of receipt

We hereby acknowledge receipt of the following submission by the proprietor:

Submission number	6932672
Application number	EP11001632.6
Patent number	EP2359834
Date of receipt	21 November 2018
Your reference	C411599EP
Proprietor	Alexion Pharmaceuticals, Inc.
Title	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

Documents submitted

package-data.xml	ep-opposition-data.xml
ep-oppo.pdf (2 p.)	AUXREQ-1.pdf\Track Amended Auxiliary Request 1 C411599EP ALS PXT.pdf (1 p.)
AUXREQ-2.PDF\Auxiliary Request 1 C411599EP ALS PXT.PDF (1 p.)	AUXREQ-3.pdf\Track Amended Auxiliary Request 2 C411599EP ALS PXT.pdf (1 p.)
AUXREQ-4.PDF\Auxiliary Request 2 C411599EP ALS PXT.PDF (1 p.)	OTHER-2.PDF\Consolidated List of Documents.PDF (2 p.)
OTHER-1.pdf\Submission under R116 C411599EP ALS PXT.pdf (7 p.)	Other-evidence-3.PDF\D41 Alberts et al (1994) page 585 - C411599EP.PDF (3 p.)
Other-evidence-2.pdf\D39 Klibanov Declaration C411599EP.pdf (139 p.)	

Submitted by	CN=Elizabeth Davenport 55598

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Correction by the EPO of errors in debit instructions filed by eOLF
Errors in debit instructions filed by eOLF that are caused by the editing of Form 1038E entries or the continued use of outdated software (all forms) may be corrected automatically by the EPO, leaving the payment date unchanged (see decision T 152/82, OJ EPO 1984, 301 and point 6.3 ff ADA, Supplement to OJ EPO 10/2007).

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Acknowledgement of receipt

We hereby acknowledge receipt of the following submission by the opponent:

Submission number	6931208
Application number	EP11001632.6
Patent number	EP2359834
Date of receipt	21 November 2018
Your reference	AMG17203OP
Opponent	Amgen Inc.
Title	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

Documents submitted	
package-data.xml	ep-opposition-data.xml
ep-oppo.pdf (2 p.)	OTHER-1.pdf\AMG17203OP_D39.pdf (9 p.)
OTHER-2.pdf\AMG17203OP_D40.pdf (107 p.)	OTHER-3.pdf\AMG17203OP_D41.pdf (75 p.)
OTHER-4.pdf\AMG17203OP_D42.pdf (61 p.)	OTHER-5.pdf\AMG17203OP_D43.pdf (11 p.)
OTHER-6.pdf\AMG17203OP_D44.pdf (2 p.)	OTHER-7.pdf\AMG17203OP_D45.pdf (9 p.)
OTHER-8.pdf\AMG17203OP_D46.pdf (6 p.)	OPPO.pdf\AMG17203OP_Reply to proprietors submission.pdf (24 p.)

Submitted by	CN=Hans Doerries 13689
Method of submission	Online

Date and time receipt generated	21 November 2018, 14:29 (CET)
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Samsung Bioepis Exhibit No. 1002
Page 7547

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Date: 29.11.2018

Reference	Application No./Patent No.
C411599EP PXT	11001632.6 - 1112 / 2359834

Applicant/Proprietor: Alexion Pharmaceuticals, Inc.

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
- ☐ Communication of
- ☒ Oral Proceedings on 21.01.2019

Enclosure(s):
- ☐ Letter from the proprietor of the patent of
- ☒ Letter from the opponent 01 of 19./21.11.2018, opponent02 of 20.11.2018
- ☒ Copy (copies) O1: cited documents D39, D43, D46
  O2: D39, D40

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

Europäisches Patentamt • European Patent Office • Office européen des brevets

☒ **Registered letter**

Samsung Bioepis Exhibit No. 1002
Page 7548

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November 19, 2018

Opposition against European patent EP 2 359 834
(Application No. 11 001 632.6)

Title:	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor:	Alexion Pharmaceuticals, Inc.
Opponent:	Amgen Inc.
Our Ref.:	AMG17203OP

Submission in Preparation of Oral Proceedings Scheduled for January 21, 2019:

This is to confirm that I will attend the Oral Proceedings scheduled for January 21, 2018 on behalf of Opponent 1 (Amgen Inc.).

I expect to be accompanied by Dr. Simon Geiger of my office. I furthermore expect to be accompanied by Tom Wrona, Senior Patent Counsel at Amgen's Patent Department.

Finally, I expect to be accompanied by our technical expert, Dr. Michael Treuheit. Dr. Treuheit's scientific qualifications will be apparent from his declaration (**D23**, Appendix D).

It is respectfully requested that Dr. Treuheit be permitted to make oral submissions during the Oral Proceedings on specific technical aspects of the case, in particular the different aspects of chemical and physical stability of antibody formulations, the content of and the data provided in his Declaration (**D23**), and the content of, and the data provided in the declaration of Dr. Miano (**D33**). Any oral submission by Dr. Treuheit would be made under my responsibility and control, as required by **G 4/95**.

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2 European Trademark Attorney
3 European Design Attorney
4 Patentanwalt
5 Solicitor (England & Wales)
6 U.S. Patent Attorney
7 U.S. Patent Agent
8 Rechtsanwalt

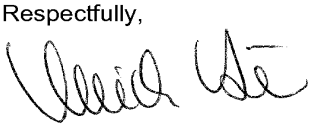
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PR-Nr. 1278

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Any submissions made during Oral Proceedings on behalf of Opponent Amgen Inc., including any possible submissions by our experts, will be in English.

Assuming that the Proprietor's and the Co-Opponent's representatives will likewise make their submissions in English, no translation will be required.

Respectfully,



Dr. H. Ulrich Dörries
European Patent Attorney

UD/KS

Samsung Bioepis Exhibit No. 1002
Page 7550

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Munich, November 20, 2018

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European Trademark Attorneys
European Design Attorneys

Our Ref.:	**198 441 u6/m25/ne** **Eculizumab Composition / ALEXION**
Opposition to:	**EP 2 359 834 B1 (11001632.6)**
Proprietor:	**Alexion Pharmaceuticals, Inc.** **Cheshire, CT 06410 (US)**
Opponent:	**Hoffmann Eitle** **Patent- und Rechtsanwälte PartmbB** **Arabellastr. 30** **81925 Munich (DE)**

The following observations are made in response to the preliminary opinion of the Opposition Division enclosed with their summons to oral proceedings issued on July 5, 2018.

Partnerschaftsregister Amtsgericht München | PR 1372
Eine Liste der Partner i.S.d. PartGG ist in unseren Büros und unter www.hoffmanneitle.com/partner einsehbar.
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Samsung Bioepis Exhibit No. 1002
Page 7551

1. REFORMULATION OF TECHNICAL PROBLEM AND NEW EXPERIMENTAL DATA ARE NOT ADMISSIBLE

(1) The parties have agreed that **D1** represents the closest prior art. The OD sees no reasons to differ (cf. point 9 of the preliminary opinion). Starting from **D1**, the proprietor has reformulated the technical problem which is solved by the teaching of the opposed patent to be

> *"the ideal balance of properties for use in the treatment of PNH, including the **enhanced stability** and thus maintenance of biological integrity following prolonged storage in the absence of preservative, whilst also providing a low-volume liquid formulation having acceptable infusion time and which is convenient to store."*
> (cf. submission of March 22, 2018; point 5.11.1; emphasis added)

(2) To demonstrate that the above technical problem is solved, the proprietor provides post-published experimental evidence by Dr. Miano (**D33**). In the proprietor's opinion, the experiments of **D33** would show that the eculizumab formulation at 10 mg/ml is sufficiently stable for a period of 48 months. Notably, **D33** is only concerned with long term stability of the eculizumab preparation. No data were provided to support the other alleged benefits / advantages, such as "*maintenance of biological integrity*" or an "*acceptable infusion time*".

(3) It is undisputed that the original specification contains no disclosure at all with respect to the stability of the claimed eculizumab formulation, let alone that this aspect is mentioned or merely suggested anywhere.

(4) Still, the proprietor is of the opinion that the new stability data can be used as a basis to reformulate the objective technical problem. Specifically, he points to case law (as laid down in section G-VII-11 of the Guidelines and **T184/82**), according to which it is possible to rely on new effects, provided that the skilled person would recognize

Samsung Bioepis Exhibit No. 1002
Page 7552

these effects as implied by or related to the problem initially suggested. We disagree for the following reasons:

(5) The sole emphasis of the original disclosure was on the improvement of "*the quality of life of a patient*" and "*prolonging the health-adjusted life expectancy or a patient"* suffering from PNH" (cf. [0006] to [0010] of the opposed patent). Said "quality of life" and its measurement is defined in more detail in sections [0044] to [0052]. All the examples exclusively deal with clinical efficacy in the context of the improving of the patient's "*quality of life*".

(6) The proprietor now argues that the technical problem solved by the invention is to be seen in the enhancement of stability of the eculizumab formulation. We submit that this technical significance concerning the allegedly improved stability of a liquid eculizumab formulation is **not derivable** from the statements on quality of life of the patients made in the patent application. Moreover, even in the proprietor's opinion, this effect is not self-evident: he himself has indicated that the increased long-term stability was surprising.

(7) Further guidance with regard the derivability of a new technical effect or technical problem is provided by the established case law of the EPO. For example, in **T386/89** the board found that where a specific problem is identified in the description, the patentee may can put forward a

> *"**modified** version of the problem particularly if the issue of inventiveness has to be considered on an objective basis against a **new prior art** which **comes closer to the invention than that considered in the original patent application or granted patent specification**".*

(8) Reference is also made in this respect to the decision **T184/82** where the Board allowed a re-definition of the problem to such an extent that the skilled person

Samsung Bioepis Exhibit No. 1002
Page 7553

"*could recognise the same as implied or related to the problem initially suggested*" (cf. point 5 of the reasons).

(9) The opposition division will appreciate that **D1** is *de facto* **no** new prior art, as it would have been required by the above case law to allow reformulation of the technical problem. As outlined in our initial opposition brief (cf. point 3.2.2), **D1** basically comprises the same disclosure as **Hillmen et al**., N Engl. J Med **2004**; 350: 552-559 **(D3)**, i.e. the latter document could likewise have served as the closest prior art. **D3** is a scientific publication corresponding to the clinical study mentioned in the Examples of **D1** and describes the details of the procedure and the results of the study in more detail.

(10) Said **Hillmen 2004** reference is cited in the description of the opposed patent (cf. **ref. 12** on page 16; 4th document in left hand column on page 31). Referring to **D3,** it is stated in [0003]:

> *"In a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements.[12] However, this unblinded study involved a small number of patients with no control arm and without protocol-driven transfusion standards."*

(11) Section [0003] is immediately followed by the chapter "Summary of the Invention". This makes it clear that the inventors have not only carefully considered **D1/D3**, but also acknowledged it as the closest prior art document. Accordingly, they have formulated the technical problem to overcome potential deficiencies of the prior art study, such as a small number of patients, no control arm, and no protocol-driven transfusion standards. All this concerns "***quality of life***" of the patients, but not at all stability of the eculizumab formulation. Only with unallowable hindsight one could assume that improvement of long-term stability should be the "objective" technical problem to be solved by the claimed teaching.

Samsung Bioepis Exhibit No. 1002
Page 7554

(12) According to the above decisions, only if the technical the problem can be clearly deduced by the skilled person from the application as originally filed when considered in relation to the nearest prior art, reformulation of the problem would not contravene **Art. 123(2) EPC**, that is to say would not constitute added subject-matter.

(13) In this regard it is noteworthy that the whole TRIUMPH study underlying the opposed patent only lasted about **6 months** (cf. [0005], [0070], referring to a "*26 week treatment period*"). The proprietor now relies on a technical effect which – if at all – may only be visible **and** technically relevant **after two or three years** of storage of the formulation. Hence it would not have objectively been possible to identify the present technical problem of long-term stability from the present application, let alone from the closest prior art.

(14) Applying the above principles, we submit that the alleged unexpected effect, i.e. the allegedly improved long-term stability, is not deducible from the application as originally filed. It follows that it cannot be taken into account when determining the problem underlying the invention for the purpose of assessing the issue of inventive step. The post-published data of **D33** should not be admitted into the proceedings.

2. LACK OF INVENTIVE STEP (ARTICLES 100(A) AND 56 EPC)

(15) It is maintained that claim 1 of the opposed patent is obvious over **D1** (or **D3**) in combination with **D11,** or common general knowledge as reflected by **D10**.

2.1 D33 MERELY CONFIRMS COMMON KNOWLEDGE OF SKILLED PERSON BEFORE THE FILING DATE

(16) In **D33**, Dr. Miano provides data, allegedly demonstrating the improved stability of an eculizumab formulation at 10 mg/ml compared to 30 mg/ml. As outlined above, the new experiments should not be admitted into the proceedings and therefore not be

Samsung Bioepis Exhibit No. 1002
Page 7555

taken into account for the assessment of inventive step. However, even if the Opposition Division considers the data relevant, they are not sufficient to support inventive step. To explain in more detail:

(17) It was common general knowledge before the filing date of the opposed patent that increasing the concentration of antibodies increases the aggregation tendency of the protein, and thus decreases its stability. In fact, there is an almost linear relationship between an increase in concentration and aggregate formation. This is reflected, e.g., by the review article **D20**:

> *"Increasing the concentration of antibodies often increases the aggregation tendency of the protein. It was demonstrated that increasing the IgG1 concentration (in 10 mM citric acid, 100 mM NaCl pH 5.5) from 2.7 mg/mL to 50 mg/mL almost linearly increased the Nephelometric Unit (NU) from 2 to 40.[52]"* (p. 9, left col., 3rd para. of **D20**)

(18) Hence, it was entirely predictable that an antibody composition formulated at 10 mg/ml shows less protein aggregation (i.e. a higher monomer content) than a composition formulated at 30 mg/ml. The data presented in **D33** merely confirm this trivial fact, which would have been obvious to the skilled person before the filing date of the opposed patent.

(19) In fact, the data submitted with **D33** illustrate the "***try-and-see***" approach the Proprietor has taken to find a concentration of 10 mg/ml. According to Dr. Miano, *"one of the stability parameters that the commercial formulation of eculizumab must meet in order to be deemed satisfactorily stable and thus suitable for release into the market is the percent of monomer antibody in the sample must be greater than or equal to 98.0%, which means that there must be less than or equal to 2.0% aggregates of the antibody […]"* (Item 3 of **D33**). In his submission, the Proprietor confirms that at least 98% monomer content represents a commercial requirement (cf. Item 5.3.4 of the Proprietor's submission dated March 22, 2018).

Samsung Bioepis Exhibit No. 1002
Page 7556

(20) Having the commercial requirement of 98% monomer content in mind, and knowing that aggregate formation of antibodies increases with increasing concentration, it was a routine task for the skilled person to test a variety of antibody concentrations, and determine the storage time after which the monomer content reaches the threshold level of 98%. **D35** shows that antibody concentrations between 0.5 mg/ml and 50 mg/ml were routinely used for pharmaceutical formulations before the filing date of the opposed patent, and thus would have been included in the stability tests conducted by the skilled person.

(21) In **D33**, Dr. Miano describes the exact kind of stability studies that would have been routine to the skilled person. However, eculizumab formulations at merely two (standard) antibody concentrations were used, i.e. at 10 and 30 mg/ml. For Figure 1, these formulations were incubated at 23-27°C, in order to simulate the degradation that would occur over an extended period of time at lower temperatures. For Figure 2, the formulations were incubated at 2-8°C, which is the recommended storage temperature for eculizumab. The monomer content was assessed at various time points. However, in the experiment conducted at 2-8°C the last time point for the concentration of 10 mg/ml was taken after 24 months, whereas for the concentration of 30 mg/ml the last time point was taken after only 18 months. Any information relating to later time points is only based on extrapolation, and not on actual data, as confirmed by Dr. Miano (cf. last sentence of Item 7 of **D33**).

(22) Notably, in both of Figures 1 and 2 of **D33**, only single data points for each time point are shown for the formulation at 30 mg/ml, suggesting that only a single batch was analysed. This is contrary to the international guidelines for stability testing of biological product, which recommend providing stability information on at least 3 batches (cf. Item 4.3 of **D39**), and casts serious doubts on the reliability of any of the data for a concentration of 30 mg/ml. **D39** is filed in direct response to the Proprietor's new technical arguments regarding the stability of the claimed eculizumab composition. Thus, should the Proprietor's new technical arguments be

Samsung Bioepis Exhibit No. 1002
Page 7557

admitted into the proceedings (which we object to; cf. Item 1 above), also **D39** should be admitted.

(23) Additionally, from Dr. Miano's statements in **D33** and the presented data it appears that the data for the 10 mg/ml and 30 mg/ml samples were not generated in side-by-side experiments. In Item 4 of **D33**, Dr. Miano indicates that a 30 mg/ml formulation was generated, but there is no mentioned of preparing a 10 mg/ml formulation. Consistently, according to **D33**, only the batch of 30 mg/ml (designated "ALXN5100") was subjected to the experimental procedure described in Item 5. It thus appears that only the data for the 30 mg/ml samples were generated specifically for **D33**. The source of the data for the 10 mg/ml samples remains elusive – they may have been generated for governmental approval of the 10 mg/ml eculizumab formulation. The notion that the 10 mg/ml and the 30 mg/ml samples have not been tested in side-by-side experiments could also explain the experimental differences outlined above, i.e. that only a single batch was tested for the 30 mg/ml concentration vs. several batches for the 10 mg/ml concentration, and that the samples at 10 mg/ml and 30 mg/ml were tested for different periods of time in Figure 2.

(24) If the data presented in **D33** were indeed not generated in side-by-side experiments, no reliable conclusions can be drawn from them. This is because it is known that various external (e.g., environmental) factors can significantly influence the results of biological experiments. These external factors differ from day to day. When experiments are then carried out over many months, as in the present case, the influence of these external factors multiplies, and severely distorts the experimental results. Merely to name one example, the temperatures at which the experiments of Figures 1 and 2 were performed are given as ranges, i.e. 23-27°C and 2-8°C, respectively. A high external temperature is likely to have shifted the actual temperature at which the experiments were performed close to the upper limit, and *vice versa*. Hence, it is entirely possible that the experiments for the 30 mg/ml samples were generated at a temperature close to 8°C, and the experiments for the

Samsung Bioepis Exhibit No. 1002
Page 7558

10 mg/ml samples at close to 2°C. Of course, such differences would have a significant impact on degradation, which is accelerated at high temperatures. For the above reasons, no reliable conclusions can be drawn from the stability studies presented in **D33**.

(25) If the data presented in **D33** are considered despite the above-described deficiencies, Figure 2 suggests that at a temperature of 2-8°C and a concentration of 30 mg/ml, the tested eculizumab formulation reached the threshold level of 98% monomer content after about 36 months of storage. Dr. Miano concludes that *"the 30 mg/ml concentration would not meet the release standards after 36 months of storage"* (Item 5.3.7 of the Proprietor's submission dated March 22, 2018). At a concentration of 10 mg/ml, the monomer content of the eculizumab formulation after 36 months of storage was about 99.5%, and thereby above the threshold level of 98%. Accordingly, a concentration of 10 mg/ml was found acceptable.

(26) However, as mentioned above, already before the filing date of the opposed patent it was common general knowledge that aggregate formation of antibodies increases almost linearly with increasing concentration (p. 9, left col., 3rd para. of **D20**). Hence, based on Figure 2, any antibody concentration between 30 mg/ml and 10 mg/ml would have a monomer content of at least 98% after 36 months of storage, and would thus be acceptable for market release. In fact, if it was desired to obtain eculizumab compositions that are even more stable than the one formulated at 10 mg/ml, the antibody concentration could simply be reduced to below 10 mg/ml. Apparently to disguise from these trivial facts, no comparative data of eculizumab compositions formulated at any other concentrations, e.g. at 20 mg/ml or below 10 mg/ml are included in **D33**.

(27) Overall, the above demonstrates that the skilled person was in a classical "try and see" situation when finding a suitable concentration for formulating eculizumab. According to established case law, a "try and see" situation is considered to have

Samsung Bioepis Exhibit No. 1002
Page 7559

occurred if the skilled person, in view of the teaching in the prior art, had already clearly envisaged a group of compounds or a compound (here: standard concentrations) and then determined by routine tests whether such compound/s (here: concentrations) had the desired effect (cf. I.D.7.2 of the EPO Case Law of the Boards of Appeal).

(28) For example, in the case underlying **T1241/03**, the board considered the problem underlying the patent in suit to be the provision of an aqueous formulation of human growth hormone which remains stable during long-term storage. The Proprietor argued that that a skilled person, knowing from the prior art that the development of stable aqueous protein formulations was a risky business, would not have had a reasonable expectation of success to arrive at the claimed subject-matter upon combination of the teaching in two prior art documents. However, the board pointed out that a reasonable expectation of success does not require certainty. Thus, the board held that in spite of the understandable uncertainties which always characterise experiments using biologic compounds like proteins, the skilled person had no reason to adopt a sceptical attitude. He or she would have had either some expectations of success or, at worst, no particular expectations of any sort, but only a "try and see" attitude, which does not equate with the absence of an reasonable expectation of success (cf. Item 32 of the “Reasons for the Decision” of **T1241/03**). Accordingly, the board denied an inventive step.

(29) The present case is comparable to the case underlying **T1241/03**. However, in the present case the skilled person did not even have to combine the teachings of different prior art documents. Rather, the skilled person could simply rely and his or her common general knowledge that aggregate formation of antibodies increases with increasing concentrations. Accordingly, faced with the problem of providing a stable eculizumab formulation, he or she would have tested a number of standard concentrations and would have found that concentrations lower than 30 mg/ml, e.g. 10 mg/ml, provide for the desired stability. Just like in **T1241/03**, the skilled person

Samsung Bioepis Exhibit No. 1002
Page 7560

had no reason to be sceptical that one of the tested antibody concentrations would solve the problem of providing a stable eculizumab formulation, i.e. a formulation with a monomer content of at least 98% after 36 months of storage at 2-8°C.

(30) Additionally, it is important to consider that no particular advantage of an eculizumab concentration of 10 mg/ml has been demonstrated by the Proprietor. As outlined above, based on Figure 2 any antibody concentration between 30 mg/ml and 10 mg/ml would have a monomer content of at least 98% after 36 months of storage, and would thus be acceptable for market release. Thus, the claimed antibody concentration of 10 mg/ml eculizumab represents an entirely arbitrary selection.

(31) Overall, faced with the problem of providing a stable eculizumab formulation, the skilled person was in a classic "try and see" situation. He or she would have conducted standard stability studies, such as the ones presented in **D33**. The data shown in **D33** are unreliable but, if anything, they merely confirm the skilled person's expectation that formulations are more stable at low antibody concentrations than at high concentrations. In accordance with **D33**, when performing standard stability studies the skilled person would have realized that any antibody concentration lower than 30 mg/ml would be acceptable for market release. The specific concentration of 10 mg/ml represents an arbitrary selection which is not associated with any unexpected technical effect. It is established case law of the EPO that a technical effect can only corroborate the patentability of an invention if it is unexpected or surprising. Thus, even if considered, the data of **D33** do not support an inventive step.

2.2 10 MG/ML IS A TYPICAL ANTIBODY CONCENTRATION ALSO FOR NON-IGG1 ANTIBODIES

(32) In his submission dated March 22, 2018, the Proprietor argues that based on the presented evidence, e.g. **D20**, it cannot be concluded that 10 mg/ml represents a

Samsung Bioepis Exhibit No. 1002
Page 7561

typical antibody concentration in pharmaceutical formulations. To support his arguments, the Proprietor refers to **D35**, in which he presents an overview of all antibodies listed in **D20** and **D21**. Strikingly, even according to **D35**, which was prepared by the Proprietor himself, the concentration of 10 mg/ml represents the single most frequent concentration among all of the listed antibody solutions. Hence, the evidence presented by the Proprietor himself supports the view that 10 mg/ml represents a typical antibody concentration in pharmaceutical formulations, which the skilled person certainly would have considered also for eculizumab formulations.

(33) In **D35**, the Proprietor has additionally included information on the isotypes of the listed antibodies. According to **D35**, eculizumab is an IgG2/4 antibody, whereas most of the other antibodies included in the list are of the IgG1 isotype. IgG2/4 antibodies were known before the filing date of the opposed patent, as evidenced e.g. by **D40**. However, the Proprietor alleges that due to the structural differences between non-IgG1 and IgG1 antibodies, no conclusions for a suitable antibody concentration for eculizumab formulations can be drawn from prior art relating to IgG1 antibodies. Moreover, the Proprietor argues that none of the five non-IgG1 antibodies in **D35** were formulated at a concentration of 10 mg/ml. Based on these observations, the Proprietor alleges that, before the filing date of the opposed patent, a concentration of 10 mg/ml was not typical for non-IgG1 antibodies.

(34) We refute the Proprietor's allegation that a concentration of 10 mg/ml was not typical for pharmaceutical non-IgG1 antibody formulations before the filing date of the opposed patent. To support our view, we herewith submit prior art documents **D41** to **D43**, all of which demonstrate that a concentration of 10 mg/ml was also common for non-IgG1 antibodies. **D40** to **D43** are filed in direct response to the Proprietor's allegation that no conclusions for a suitable antibody concentration of non-IgG1 antibodies can be drawn from prior art relating to IgG1 antibodies. Therefore, the documents should be admitted into the proceedings.

Samsung Bioepis Exhibit No. 1002
Page 7562

(35) Additionally, it should be considered that the opposed patent does not mention any significance of the antibody isotype for the stability of eculizumab formulations. The only mention of isotypes in the opposed patent is found in paragraph [0055], and relates to the recognition of IgG1 and IgG3 antibodies by phagocytic cells. The specific isotype of eculizumab (IgG2/4) is not even mentioned in the opposed patent. Hence, **D40** to **D43** are filed in response to an unpredictable technical argument by the Proprietor, and should be admitted into the proceedings also for this reason.

(36) **D41** relates to the pharmaceutical IgG4 antibody natalizumab. The particular focus of **D41** is to provide stable antibody formulations:

> *"Therefore, notwithstanding what has previously been reported in the literature, there exists a need for improved methods of formulating proteins and/or antibodies. There is also a need for stable formulations with large concentrations of antibody or protein, where the activity of the antibody or protein is retained. Also needed are stable formulations of concentrated protein which maintain a fixed volume. Applicants disclose herein stable compositions which can be further utilized to prepare antibody formulations, especially formulations with high concentrations of antibody which do not precipitate out and are stable when stored at the recommended temperatures. The highly concentrated and stable antibody formulations will greatly aid physicians in treating subjects of variable weights."* (p. 1, ll. 25-34 of **D41**)

(37) In **D41** the problem of providing a stable natalizumab composition is solved by adding phosphate buffer, polysorbate and sodium chloride. That way, pharmaceutical formulations comprising antibody concentrations between 0.1 and 200 mg/ml can be prepared (cf. p. 2, ll. 1-9 of **D41**). The concentration of 10 mg/ml is singled out on page 13, lines 2 to 5 of **D41**. Notably, Examples 11 and 12 list all ingredients of two exemplary, stable natalizumab formulations at exactly 10 mg/ml (pp. 23-24 of **D41**). Like the eculizumab composition of the opposed patent, the natalizumab compositions of **D41** are preservative-free. These data show that a

Samsung Bioepis Exhibit No. 1002
Page 7563

concentration of 10 mg/ml is a typical concentration also for non-IgG1 antibody formulations, e.g. for formulations comprising IgG4 antibodies such as natalizumab.

(38) **D42** relates to the IgG4 antibody volociximab (p. 2, ll. 6-8 and p. 8, l. 22 of **D42**). The antibody targets the α5β1 integrin and inhibits the growth of cancer cells (cf. p. 1, ll. 5-7 of **D42**). On page 20, lines 19 to 23, **D42** indicates that in a preferred embodiment, the antibody is formulated as a pharmaceutical composition comprising a solution of between about 1.0 and 15.0 mg/ml antibody. **D42** further states that *"in a particularly preferred embodiment the pharmaceutical composition comprises a solution of about 10.0 mg/mL antibody, […]"* (p. 20, ll. 24-26 of **D42**). In a table listing the parameters of a phase II clinical study, the concentration of volociximab compositions for treating patients with metastatic renal cell carcinoma is indicated to be 10 mg/ml (cf. p. 41, "Dosage Form and Strength/Formulation" of **D42**). Hence, also **D42** demonstrates that a concentration of 10 mg/ml is a typical concentration for non-IgG1 antibody formulations.

(39) Finally, **D43** relates to antibodies that regulate the activity of NK cells. Although the preferred antibody is of the IgG4 isotype, reference is also made to IgG1, IgG2 and IgG3 antibodies (cf. claims 15-16 of **D43**). The preferred pharmaceutical composition comprising these antibodies has a concentration of 10 mg/ml:

> *"For example, an antibody present in a pharmaceutical composition of this invention can be supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials."* (p. 73, ll. 17-19 of **D43**)

(40) Overall, **D35** as presented by the Proprietor himself in combination with **D41**, **D42** and **D43** show that, before the filing date of the opposed patent, a concentration of 10 mg/ml was not only typical for pharmaceutical formulations of IgG1 antibodies, but also common for non-IgG1 antibodies such as IgG4. In view thereof, the skilled

Samsung Bioepis Exhibit No. 1002
Page 7564

person would certainly have considered a concentration of 10 mg/ml for the IgG2/4 antibody eculizumab, e.g. when performing stability studies as outlined above.

3. CONCLUSION

(41) The data contained in **D33** should not be admitted into the proceedings. However, even if admitted, they do not support an inventive step. Moreover, before the filing date of the opposed patent, formulating antibody compositions at 10 mg/ml was common in the field, also for non-IgG1 antibodies. Thus, as laid out in our notice of opposition dated August 9, 2017, all of the features in combination as claimed in the opposed patent were obvious over **D1** (or **D3**) in combination with **D11,** or common general knowledge as reflected by **D10**.

(42) Consequently, the opposed patent does not meet the requirements of the EPC. It has therefore to be revoked *in toto*.

(43) We will use the English language in the Oral Proceedings on January 21, 2019. A translation from English into German is not required. Should any other language besides English be used by any party, translation into English is required.



DR. LEO POLZ

European Patent Attorney
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Samsung Bioepis Exhibit No. 1002
Page 7565

Enclosures:

D39 ICH Harmonised Tripartite Guideline on Stability Testing of Biological Products

D40 Mueller et al. (1997): Humanized porcine VCAM-specific monoclonal antibodies with chimeric IgG2/G4 constant regions block human leukocyte binding to porcine endothelial cells. Mol Immunol. 34(6): 441-452.

D41 WO 2004/071439 A2

D42 WO 2005/092073 A2

D43 WO 2006/003179 A2

Samsung Bioepis Exhibit No. 1002
Page 7566

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HUMANIZED PORCINE VCAM-SPECIFIC MONOCLONAL ANTIBODIES WITH CHIMERIC IgG2/G4 CONSTANT REGIONS BLOCK HUMAN LEUKOCYTE BINDING TO PORCINE ENDOTHELIAL CELLS

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Abstract—Discordant xenografts surviving the initial hyperacute rejection phase may be subject to cellular rejection processes mediated by infiltrating leukocytes including T cells, NK cells and monocytes. The stable adhesion of these cell types to endothelial cells is due to the molecular interaction of the integrins VLA-4 and LFA-1 with their ligands vascular cell adhesion molecule (VCAM) and ICAM-1 present on the endothelial cells. Human VLA-4 binds to porcine VCAM, and blocking mAbs specific for porcine VCAM have been developed. We have localized the epitope of the anti-porcine VCAM blocking mAbs 2A2 and 3F4 to domains 1 and 2, respectively. Humanized antibodies (IgG4 isotype) were constructed from these anti-porcine VCAM antibodies and demonstrated to inhibit adhesion of Ramos, Jurkat and YT cells, as well as purified resting and activated human T cells, to porcine aortic endothelial cells (PAEC). These cell types express both LFA-1 as well as VLA-4, suggesting blockade of human VLA-4 interaction with porcine VCAM may alone be sufficient to significantly impair adhesion of human leukocytes to porcine endothelial cells. The chimeric anti-porcine VCAM (pVCAM) HuG4 antibodies promoted increased adhesion of Fc receptor (FcR) positive cells such as U937 monocytic cells to PAEC. In contrast, chimeric anti-porcine VCAM antibodies created using the CH1 and hinge region from human IgG2 and the CH2 and CH3 regions from human IgG4 (HuG2/G4 antibodies) inhibited binding of FcR positive cells to PAEC. These chimeric anti-pVCAM antibodies should allow delineation of the *in vivo* role of VLA-4/VCAM interaction in porcine-to-primate xenotransplants. Further, the design of the HuG2/G4 antibodies should render them efficacious in multiple settings requiring elimination of FcR binding. © 1997 Elsevier Science Ltd.

Key words: adhesion molecules, antibodies, Fc receptors, inflammation, xenotransplantation.

INTRODUCTION

Although much progress has been made in elucidating the molecular mechanisms of hyperacute rejection of xenotransplants (reviewed in Somerville and D'Apice, 1993), the mechanisms of cell mediated rejection of xenografts remain unclear. The cellular infiltrate observed 3 days following transplantation of guinea pig hearts into complement (C)-depleted rats is comprised predominantly of macrophages and natural killer cells, with low numbers of T lymphocytes present (Blakely *et al.*, 1994). Similarly, the cellular infiltrate present 6 days after transplantation of porcine fetal islet clusters into rats is comprised predominantly of macrophages with low numbers of T lymphocytes present primarily at the periphery of the graft (Wallgren *et al.*, 1995). *In vitro* porcine aortic endothelial cells (PAEC) as well as antigen presenting peripheral blood cells can directly activate human CD4+ and CD8+ T lymphocytes (Rollins *et al.*, 1994*a*; Murray *et al.*, 1994; Yamada *et al.*, 1995). Thus the initial phases of cellular rejection of xenotransplants are likely to involve binding and activation of multiple types of leukocytes including not only T lymphocytes but also natural killer cells and monocytes.

The stable adhesion of lymphocytes, natural killer cells, and monocytes to endothelial cells is mediated predominantly by the interaction of the integrins LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29) with their cognate ligands ICAM-1 and VCAM-1 on endothelial cells (Allavena *et al.*, 1991; Jonjic *et al.*, 1992). For both monocytes (Luscinskas *et al.*, 1994) and T lymphocytes (Alon *et al.*, 1995; Luscinskas *et al.*, 1995) the binding of VLA-4 to VCAM is an essential component of the *in vitro* adhesive interactions required for stable arrest under

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Samsung Bioepis Exhibit No. 1002
Page 7567

conditions of flow. *In vitro*, the transmigration of NK cells and monocytes across human umbilical vein endothelial cell (HUVEC) monolayers also utilizes both LFA-1/ICAM-1- and VLA-4/VCAM-dependent pathways (Bianchi *et al.*, 1993; Meershaert and Furie, 1994; Chuluyan *et al.*, 1995). *In vivo*, the interaction of LFA-1 with ICAM-1 may not be completely essential for extravasation of lymphocytes or monocytes since both of these cell types are present in inflammatory infiltrates of leukocyte adhesion deficiency patients who have a genetic deficiency in CD18 and thus lack integrins such as LFA-1 (Hawkins *et al.*, 1992). Finally, numerous studies have demonstrated the potential of blocking antibodies directed against LFA-1 (Issekutz, 1992; Scheynius *et al.*, 1993), VLA-4 (Issekutz, 1991; Yednock *et al.*, 1992; Chisholm *et al.*, 1993), ICAM-1 (Zeng *et al.*, 1994), or VCAM (Orosz *et al.*, 1993; Silber *et al.*, 1995) to inhibit the accumulation of lymphocytes at inflammatory sites in animals.

For xenogeneic cellular interactions it is not yet fully defined which adhesion molecule interactions will function across species barriers. It is likely that at least some xenogeneic receptor–ligand interactions will be nonfunctional. For example, mouse LFA-1 does not bind to human ICAM-1 (Johnston *et al.*, 1990). To explore the molecular basis of adhesive interactions between distinct classes of adhesion molecules on human and porcine cells we have cloned porcine E-selectin and VCAM-1 and have demonstrated that both are capable of mediating the adhesion of human leukocytes (Rollins *et al.*, 1994*a*; Mueller *et al.*, 1995). Further, we have developed murine monoclonal antibodies (mAbs) specific for porcine VCAM which block binding of human VLA-4 to porcine VCAM (Mueller *et al.*, 1995). The functional blocking activity of these antibodies suggests that they will be useful in suppressing the cellular response to a porcine xenograft. However, murine mAbs are not ideally suited for use in primates due to their short half-life and high immunogenicity (Khazaeli *et al.*, 1994). Here we have utilized antibody engineering strategies to create humanized anti-porcine VCAM antibodies that block adhesion of human natural killer cells, monocytes, and T lymphocytes to activated PAEC. Furthermore, we show the choice of human isotype has critical implications for the potential therapeutic use of humanized antibodies in xenotransplantation.

MATERIALS AND METHODS

Cells

Ramos, Jurkat and U-937 cells were obtained from the American Type Culture Collection, YT cells were the gift of Dr J. M. Wang (National Cancer Institute, Frederick, MD, U.S.A.), porcine aortic endothelial cells (PAEC) were obtained from Cell Systems (Kirkland, WA, U.S.A.) and 293-EBNA cells were obtained from Invitrogen (San Diego, CA, U.S.A.). U-937 cells were maintained in RPMI 1640 supplemented with 15% heat-inactivated FBS and 2 mM L-glutamine. Ramos and Jurkat cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. YT were maintained in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. PAEC were maintained in medium A consisting of M199 supplemented with 20% heat-inactivated FBS, 0.05 mg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.), 20 mM HEPES and 0.1 mg/ml heparin, and were used within three passages. 293-EBNA cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml G418. Human T cells were purified as described previously (Mueller *et al.*, 1995).

Production of VCAM/Ig fusions

A plasmid containing the human IgG1 Fc region was obtained from Dr M. J. Lenardo (NIH, Bethesda, MD, U.S.A.). DNA fragments containing various domains of human or porcine VCAM were obtained by PCR amplification of appropriate templates and joined in frame to the human IgG1 Fc coding region using standard molecular biology techniques. The cassettes encoding the fusion proteins were ligated into the expression plasmid pAPEX-3 (Evans *et al.*, 1995*a*). VCAM/Ig fusion proteins were produced by transfection of 293-EBNA cells as described previously (Evans *et al.*, 1995*a*). Three days following transfection the media were harvested, centrifuged to remove debris, and stored at 4°C. For ELISA, PolySorp 96-well microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 100 ng/well goat anti-human IgG Fc (Sigma, St. Louis, MO, U.S.A.) in 0.1 M carbonate buffer, pH 9.6. The plates were then washed three times with PBS supplemented with 0.5% Tween 20 and incubated with blocking buffer (PBS supplemented with 0.05% Tween 20) at 37°C for 1 hr. After three further washes, various dilutions of conditioned media in blocking buffer were added and incubated at 37°C for 1 hr. Following three further washes, 2A2 or 3F4 mAb in blocking buffer was added and incubated for 1 hr at 37°C. After additional washing, peroxidase-conjugated goat anti-mouse IgG Fc (Sigma) diluted 1:2000 in blocking buffer was added and incubated at 37°C for 1 hr. After three final washes the plate was developed for 15 min with 0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma). Reactions were stopped by addition of 1 M sulfuric acid. Quantitation was performed by measuring absorbance at 490 nm using a Bio-Rad model 3550 plate reader.

Production of chimeric antibodies

Variable regions were cloned from the 2A2 and 3F4 hybridomas by PCR amplification as described previously (Evans *et al.*, 1995*a*, 1995*b*). These sequences have been submitted to the EMBL/GenBank/DDBL under accession numbers U78799–U78802. The variable regions were joined to genomic DNA cassettes containing

Samsung Bioepis Exhibit No. 1002
Page 7568

either the intact human IgG4 constant regions or the first constant region and hinge linker region from human IgG2 followed by the second and third constant regions from human IgG4 using standard molecular biology techniques. Chimeric antibodies were produced in 293-EBNA cells and purified by chromatography on protein A Sepharose as described (Evans *et al.*, 1995*a*). Each chimeric antibody preparation was greater than 95% pure as judged by Coomassie staining of SDS polyacrylamide gels. F(ab')2 was prepared by digestion of the antibodies with ficin (Pierce, Rockford, IL, U.S.A.) followed by protein A chromatography.

Flow cytometry

Cells used were obtained by trypsinization (PAEC and 293-EBNA) or centrifugation (Ramos, Jurkat, YT and U-937 cells). Harvested cells were washed once with 4°C PBS containing 1% heat-inactivated FBS (PBS/1) and aliquoted at 50 000–100 000 cells per well in 96-well V-bottom plates (Nunc). Cells were washed two additional times with PBS/1, and then incubated with PBS/1 containing 10 μg/ml primary Ab or PBS/1 alone for 30 min at 4°C. For some experiments the chimeric HuG4 or HuG2/G4 was centrifuged at $1 \times 10^5 g$ for 1 hr prior to addition to the cells. The cells were then washed twice with PBS/1 and resuspended in PBS/1 containing the appropriate FITC-conjugated secondary antibodies (1:200 dilution) for 30 min at 4°C. The cells were again washed twice with PBS/1 and resuspended in PBS. Cell fluorescence was determined using a Becton Dickinson FACSort.

Adhesion assays

On day 0 PAEC were plated in medium A at a density of 10 000 cells per well in a 96-well flat bottom plate. On day 1 the PAEC were refed medium A supplemented with 1 μg/ml LPS (Sigma, *E. coli* 0111:B4). On day 2, human cells were resuspended in the appropriate growth medium supplemented with 10 μM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) and incubated at 37°C for 30 min. The cells were subsequently washed three times with growth medium and resuspended at 6×10^6 cells/ml in RPMI 1640 supplemented with 1% heat-inactivated FBS (RPMI/1). The PAEC were washed twice with RPMI/1 and refed 50 μl/well of RPMI/1 containing the indicated concentrations of antibodies. After adding calcein-labeled cells at a density of 3×10^5 cells/well (50 μl), the plate was centrifuged at 200*g* for 1 min and incubated at 37°C for 15 min. The nonadherent cells were removed by washing three to five times with RPMI/1. Adherent cells were lysed with HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Background fluorescence was determined from wells containing PAEC that did not receive calcein-labeled cells. Samples were performed in duplicate in each experiment. Adherence was determined by comparing the residual fluorescence concentration in each well to the input fluorescence concentration. In each experiment the specific fluorescence signal obtained in the absence of competing antibody was defined as 100%.

For adhesion assays using purified soluble pVCAM (Mueller *et al.*, 1995), PolySorp 96-well microtiter plates (Nunc) were coated overnight at 4°C with 200 ng/well purified soluble pVCAM in 0.1 M carbonate buffer, pH 9.6. After blocking the plate with RPMI 1640 supplemented with 10 mg/ml BSA (RPMI/BSA), the plates were incubated with RPMI/BSA containing antibody for 1 hr at 37°C. Subsequently, 3×10^5 calcein-labeled U-937 cells in RPMI/BSA were added to each well. The plate was centrifuged upright for 1 min at 200*g* and incubated at 37°C for 15 min. The plate was then centrifuged inverted at 200*g* for 3 min. Adherent cells were lysed by the addition of HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

RESULTS

Identification of porcine VCAM domains recognized by blocking mAbs

Previously, we have identified two murine mAbs (3F4 and 2A2) directed against porcine VCAM (pVCAM) which block VLA-4 dependent adhesion of peripheral blood human T cells to PAEC. These two mAbs are specific for pVCAM and do not recognize human VCAM (hVCAM). Here an initial analysis of the epitope specificity of the 2A2 and 3F4 mAbs was performed using fusion proteins consisting of various domains of pVCAM or hVCAM joined to the Fc region of human IgG1. The VCAM/Ig fusion proteins were produced in 293-EBNA cells and assayed for binding of 2A2 and 3F4 mAbs by ELISA (Fig. 1). Both the 2A2 and 3F4 mAbs bound efficiently to proteins containing the first three domains of pVCAM. The 2A2 mAb bound to fusion proteins containing pVCAM domain 1 such as pD1 + pD2 (porcine domain 1 plus porcine domain 2) and pD1 + hD2 (porcine domain 1 plus human domain 2), but did not bind to fusion proteins lacking pVCAM domain 1 such as pD2 + pD3, hD1 + pD2, or hD1 + hD2. Weak binding of the 2A2 mAb could also be detected to a fusion protein containing only porcine domain 1 (pD1) but not to fusion proteins containing only pD2 or pD3. Conversely, the 3F4 mAb bound to fusion proteins containing pVCAM domain 2, such as pD1 + pD2, pD2 + pD3, and hD1 + pD2, but did not bind to fusion proteins lacking pVCAM domain 2, such as pD1 + hD2 and hD1 + hD2. Binding of the 3F4 mAb could not be detected to fusion proteins containing only a single pVCAM domain. The weak binding of 2A2 and 3F4 to fusion proteins containing only a single VCAM domain is similar to previously characterized blocking antibodies specific for human VCAM and ICAM-1 which require the presence of at least two adhesion molecule domains for binding (Pepinsky *et al.*, 1992; Renz *et al.*, 1994). Together, these results suggested the 2A2 mAb specificity is defined by

Samsung Bioepis Exhibit No. 1002
Page 7569

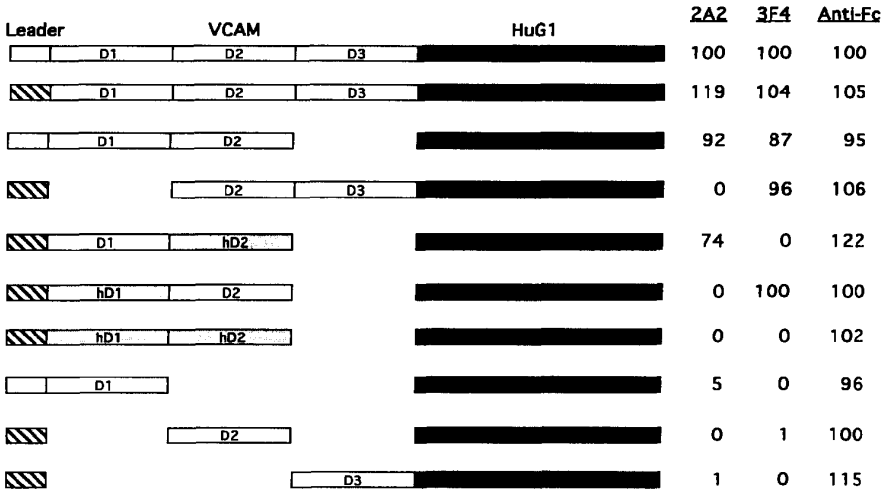


Fig. 1. Binding of 3F4 and 2A2 mAbs to VCAM/Ig fusion proteins. Proteins consisting of domains 1 (D1), 2 (D2), or 3 (D3) from porcine VCAM (open boxes) or human VCAM (gray boxes) joined to the Fc region of human IgG1 (black boxes) were produced in 293-EBNA cells. In some constructs the pVCAM secretory leader sequence was replaced with the secretory leader sequence from human CD59 (striped boxes). The fusion proteins were captured by incubating diluted conditioned media in 96-well plates coated with goat anti-human IgG (Fc specific). After incubation with 2A2 or 3F4 mAb, bound murine mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific). The total amount of each fusion protein captured per well was quantitated using peroxidase-conjugated goat anti-human IgG (Fc specific). The absorbance signal obtained with the fusion protein containing the intact pVCAM domains 1, 2 and 3 was defined as 100% for each antibody. Binding of the 2A2 and 3F4 mAbs to the remaining fusion proteins is reported as a percentage of this value. Results shown are from a representative experiment of several performed.

pVCAM domain 1 whereas the 3F4 mAb specificity is defined by pVCAM domain 2.

Inhibition of human leukocyte binding to PAEC by chimeric anti-porcine VCAM mAbs

The use of murine antibodies in porcine-to-primate xenotransplantation models or ultimately in porcine-to-human xenotransplants poses several problems including a short half-life of the murine antibody plus development of an immune response directed against the murine antibody. These problems have been significantly reduced by the use of chimeric antibodies consisting of the murine variable regions joined to human constant regions (Khazaeli *et al.*, 1994). To create anti-pVCAM chimeric antibodies we cloned the variable regions from both 2A2 and 3F4. The murine variable heavy regions were joined to the human IgG4 constant region (HuG4) since this isotype does not activate complement (C) (Tao *et al.*, 1993), the predominant mediator of hyperacute rejection of xenografts. The murine variable light regions were joined to the human kappa constant region. Chimeric ch2A2 HuG4 and ch3F4 HuG4 antibodies were produced by coexpression of the chimeric heavy and light chains in 293-EBNA cells and tested for binding to pVCAM by FACS analysis using 293 cells expressing pVCAM (Fig. 2(A)). Both chimeric mAbs produced identical staining profiles as the corresponding murine mAbs, while neither chimeric mAb bound to untransfected 293 cells. Additionally, both the chimeric antibodies and murine mAbs produced identical FACS profiles using LPS-activated porcine aortic endothelial cells (Fig. 2(B)), confirming the chimeric antibodies retained the ability to bind to pVCAM expressed on PAEC.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to block the interaction between human VLA-4 and porcine VCAM was assessed by measuring inhibition of Ramos cell binding to activated PAEC (Fig. 3). The murine 3F4 mAb inhibited this binding by 90% when present at concentrations greater than 5 μg/ml. A control humanized antibody directed against human C5 (h5G1.1 HuG4) did not inhibit the binding of Ramos to activated PAEC. The ch2A2 HuG4 and ch3F4 HuG4 produced nearly identical inhibition curves as the murine 3F4 mAb, demonstrating the retention of binding avidity and cell adhesion blocking capabilities in these chimeric antibodies.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 mAbs to inhibit binding of cells expressing both VLA-4 and LFA-1 was next examined using the Jurkat T cell line. Both the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of Jurkat to activated PAEC by up to 90% while the control antibody h5G1.1 HuG4 did not inhibit binding at any concentration (Fig. 4(A)). Similarly, the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of both resting (Fig. 4(B)) and activated (Fig. 4(C)) T cells to activated PAEC, although the

Samsung Bioepis Exhibit No. 1002
Page 7570

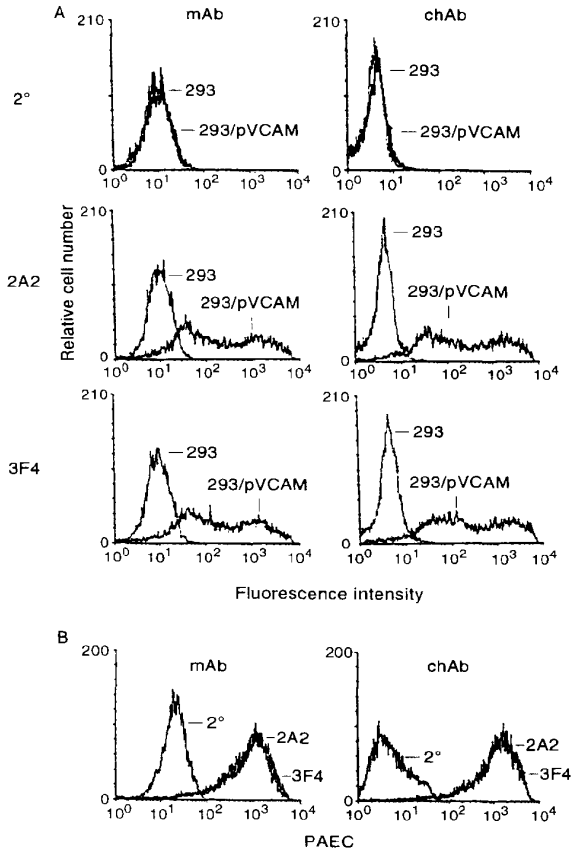


Fig. 2. Binding of 3F4 and 2A2 chimeric antibodies to pVCAM. (A) Chimeric mAbs containing the variable regions of either 2A2 or 3F4 joined to human IgG4 and kappa constant regions were produced in 293-EBNA cells and purified by protein G Sepharose chromatography. Untransfected 293-EBNA cells (293) or 293-EBNA cells expressing pVCAM (293/pVCAM) were incubated with 10 μg/ml purified murine 2A2 mAb, murine 3F4 mAb, ch2A2 HuG4, or ch3F4 HuG4. Bound antibody was detected using either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-human IgG followed by flow cytometry using a Becton Dickinson FACSort. Data are displayed as histograms with fluorescence intensity plotted on the *x*-axis and relative cell number plotted on the *y*-axis. Background staining by the secondary antibodies is shown in the panels labeled 2°. (B) PAEC were activated by treatment with 1 μg/ml LPS for approximately 16 hr. Cells were then stained with either the murine 2A2 and 3F4 mAbs (left panel) or the ch2A2 HuG4 and ch3F4 HuG4 antibodies (right panel). Background staining by the secondary antibodies (2°) is indicated.

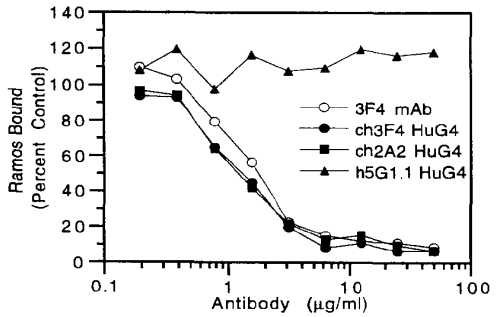


Fig. 3. Binding of Ramos cells to PAEC in the presence of mAbs. On day 0, PAEC were plated at 10 000 cells per well in medium A in 96-well plates. On day 1 the PAEC were activated by adding fresh medium A supplemented with 1 μg/ml LPS. After 16 to 24 hr incubation, the PAEC were washed and refed RPMI 1640 containing 1% FCS plus the indicated concentrations of the murine 3F4 mAb (3F4 mAb), the chimeric 2A2 and 3F4 mAbs (ch2A2 HuG4 and ch3F4 HuG4) or a humanized negative control antibody (h5G1.1 HuG4). Calcein-labeled Ramos cells were added and allowed to adhere to the PAEC under static conditions for 15 min. After nonadherent cells were removed by washing, the remaining bound cells were lysed with SDS. Calcein fluorescence of the SDS supernatant was measured with a Millipore CytoFluor 2350. In each experiment the binding of Ramos cells to PAEC in the absence of antibody was defined as 100%. Results shown are the mean of two to four experiments, with each value determined in duplicate in each experiment.

maximal inhibition achieved was reduced to approximately 60%. Addition of a blocking mAb directed against human VLA-4 also maximally inhibited adhesion by 60% (Fig. 4(C)), confirming the binding inhibition observed was due to blockade of the molecular interaction between human VLA-4 and porcine VCAM.

The potential of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to inhibit binding of natural killer cells and monocytes to activated PAEC was examined using the cell lines YT (Yodio *et al.*, 1985) and U-937 (Sundstron and Nilsson, 1976), respectively. Binding of YT cells to activated PAEC was strongly inhibited by concentrations of 10 μg/ml ch2A2 HuG4 and ch3F4 HuG4 antibodies (Fig. 5(A)). In contrast, the ch2A2 HuG4 and ch3F4 HuG4 antibodies did not inhibit binding of U-937 cells to activated PAEC (Fig. 5(B)). One possible explanation for this failure to inhibit U-937 cell binding could be the presence of elevated levels of VLA-4 on U-937 cells. However, FACS analysis revealed the presence of comparable levels of CD49d and activated CD29 on U-937 cells as on other cell lines such as Jurkat whose adhesion to PAEC was inhibited by the chimeric anti-pVCAM antibodies (Fig. 6). Interestingly, the adhesion of U-937 to PAEC was consistently augmented by approximately 50% in all experiments. This increase was specific for the anti-pVCAM antibodies as no increase in U-937 binding to PAEC was detected in the presence of the control h5G1.1 HuG4 antibody (Fig. 5(B)). This suggested that binding of U-937 to PAEC could be increased through FcR binding to the anti-pVCAM HuG4 antibodies on the PAEC surface. In support of this hypothesis, FACS analysis confirmed the presence of significant levels of FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) on the surface of U-937 cells. In contrast, Jurkat and YT cells had no detectable Fcγ receptors while Ramos cells showed the presence of only low levels of FcγRII (Fig. 6).

Binding of the HuG4 chimeric antibodies to U-937 could be directly demonstrated by flow cytometry (Fig.

Samsung Bioepis Exhibit No. 1002
Page 7571

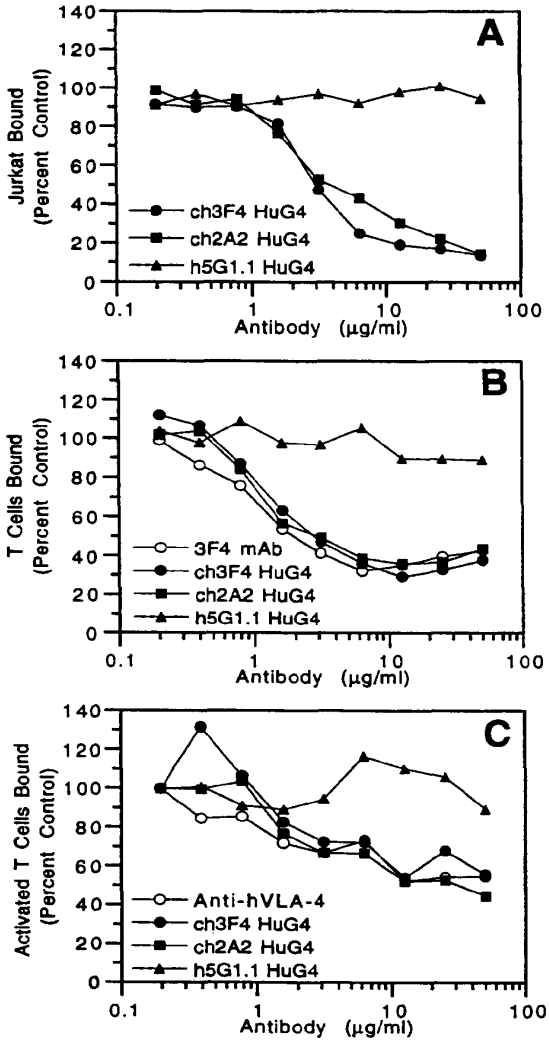


Fig. 4. Binding of Jurkat and human T cells to PAEC in the presence of HuG4 antibodies. Binding experiments were performed as described in Fig. 3 using calcein-labeled Jurkat cells (A) or calcein-labeled purified human T cells (B and C). Human T cells were either assayed immediately after purification (B) or cultured in the presence of 1 μg/ml PHA for 5 days followed by 1 day without PHA stimulation (C) prior to performing the adhesion assay. Results shown are the mean of two to four experiments (A and B) or from an individual experiment (C). Anti-hVLA-4 denotes the blocking murine mAb HP2/1 directed against the α4 subunit of human VLA-4.

7). Both the HuG4 antibodies as well as the anti-human C5 HuG4 antibody bound to U-937 cells. In contrast, 293/pVCAM cells bound only the anti-pVCAM antibodies but not the anti-human C5 HuG4 antibody. No binding of the HuG4 chimeric antibodies could be detected with Ramos, Jurkat, or YT cells. These results suggest that the HuG4 chimeric antibodies bound to U-937 cells through interaction of the HuG4 Fc region with the Fc receptors (FcRs) present on U-937 cells. Finally,

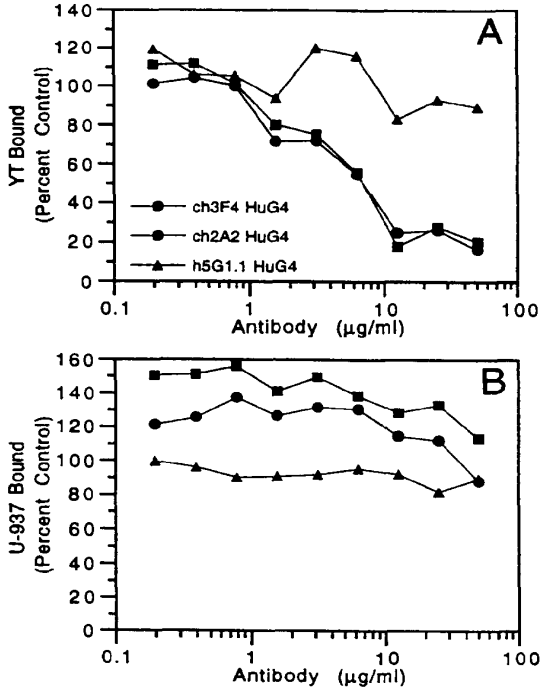


Fig. 5. Binding of YT and U-937 to PAEC in the presence of HuG4 mAbs. Adherence of calcein-labeled YT cells (A) or U-937 cells (B) to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

F(ab')2 fragments prepared from ch3F4 HuG4 strongly inhibited binding of U-937 cells to PAEC (Fig. 8) whereas F(ab')2 fragments prepared from h5G1.1 HuG4 did not inhibit the binding. This result demonstrates that, if antibody crosslinking is prevented, blockade of the interaction between human VLA-4 and porcine VCAM can effectively inhibit the adhesion of human monocytes to PAEC.

Inhibition of FcR binding of humanized mAbs with altered constant regions

Although F(ab')2 fragments proved capable of inhibiting adhesion of U-937 cells, antibody fragments are suboptimal for chronic treatments, as they have much shorter *in vivo* half-lives than intact antibodies (Covell *et al.*, 1986). The use of intact antibodies with human constant regions as anti-rejection agents is complicated by the capacity of such antibodies to fix C and/or to bind FcRs. Human antibodies of the G4 isotype are devoid of C activity (Tao *et al.*, 1993; Smith *et al.*, 1995). Human antibodies of the G2 isotype do not activate C or bind FcRs (Canfield and Morrison, 1991; Burton and Woof, 1992). Rather than altering the specific residues known to mediate C fixation and FcR binding, we explored the possibility of creating an antibody with a composite human constant region. To create chimeric mAbs which would not activate C or bind to FcRs, we utilized the overall similarity in the human G2 and G4 primary

Samsung Bioepis Exhibit No. 1002
Page 7572

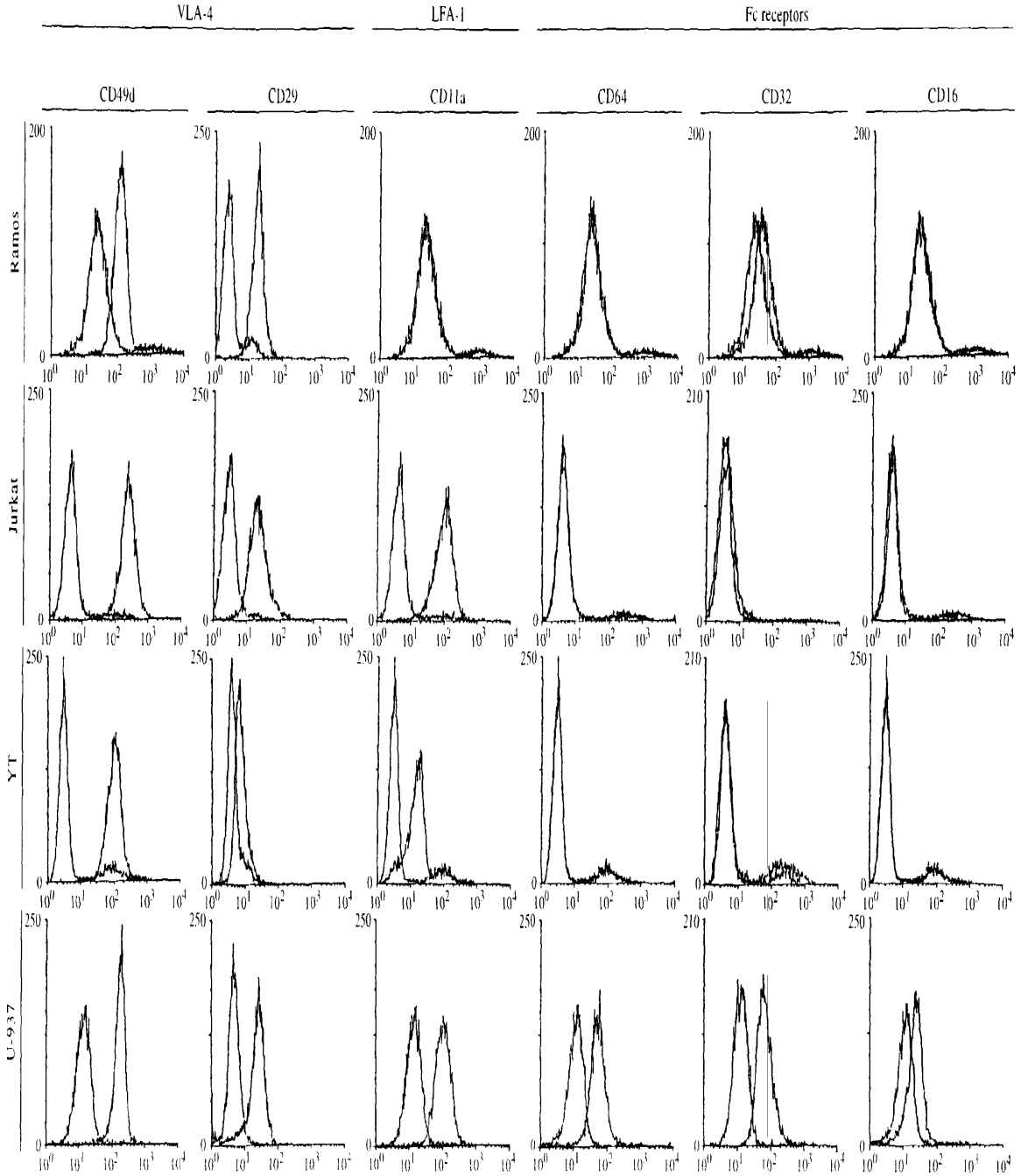


Fig. 6. Expression of VLA-4, LFA-1 and FcRs on the surface of Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml of the following primary antibodies: mouse anti-human CD49d, rat anti-activated human CD29, mouse anti-human CD11a, mouse anti-human CD64 ($Fc_\gamma RI$), mouse anti-human CD32 ($Fc_\gamma RII$) or mouse anti-human CD16 ($Fc_\gamma RIII$). After washing, cells were incubated with either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rat IgG as appropriate. Each panel presents the results obtained in the presence of primary plus secondary antibody or in the presence of secondary antibody alone.

Samsung Bioepis Exhibit No. 1002
Page 7573

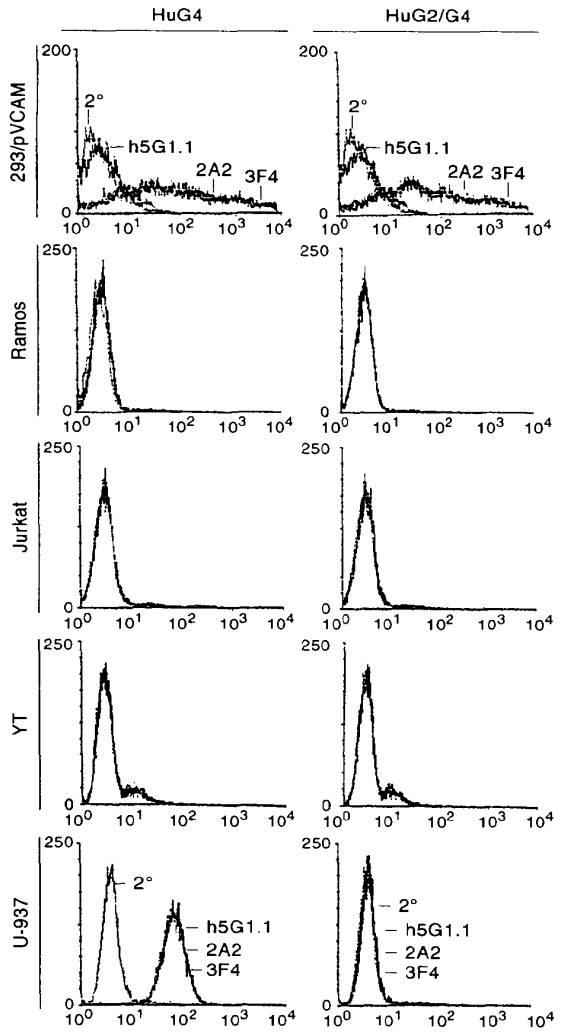


Fig. 7. Binding of chimeric antibodies to 293/pVCAM, Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml h5G1.1 HuG4, ch2A2 HuG4, or ch3F4 HuG4 (left panels) or 10 μg/ml h5G1.1 HuG2/G4, ch2A2 HuG2/G4, or ch3F4 HuG2/G4 (right panels). After washing, cells were incubated with FITC-conjugated goat anti-human IgG except for Ramos cells which were incubated with FITC-conjugated goat anti-human IgG (γ chain specific). Each panel presents the results obtained with the indicated cell line for h5G1.1 HuG4, ch2A2 HuG4 and ch3F4 HuG4 mAbs as well as the secondary antibody alone (2°) control.

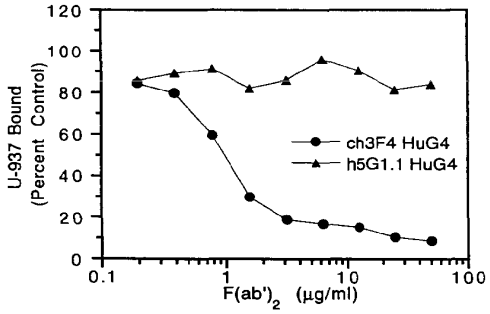


Fig. 8. Binding of U-937 cells to PAEC in the presence of F(ab')2 fragments. F(ab')2 fragments were prepared from h5G1.1 HuG4 and ch3F4 HuG4 by digestion with ficin. Undigested antibody and Fc fragments were removed by protein A chromatography. Adherence of calcein-labeled U-937 cells to PAEC in the presence of F(ab')2 was assayed as described in Fig. 3. Results shown are the mean of five experiments.

sequences to design a HuG2/G4 composite antibody consisting of the first constant region domain and the hinge region from the HuG2 isotype joined to the second and third constant region domains from the HuG4 isotype (Fig. 9). The resulting mAb did not contain the antibody sequences necessary for FcR binding, nor any new epitopes that would likely be immunogenic.

Binding to FcR was examined by using the monocytic cell line U937. FACS analysis demonstrated that the resulting ch3F4 HuG2/G4, ch2A2 HuG2/G4 and h5G1.1 HuG2/G4 antibodies did not bind to U-937 cells (Fig. 7). The ch3F4 HuG2/G4 and ch2A2 HuG2/G4 antibodies maintained the ability to bind pVCAM as demonstrated both by binding to 293/pVCAM cells (Fig. 7) and by inhibition of Jurkat and YT binding to activated PAEC (Fig. 10). For both Jurkat and YT cells inhibition of binding with the chimeric HuG2/G4 was equivalent to the HuG4 or original murine anti-pVCAM antibodies. The binding of U-937 cells to PAEC was now potently inhibited by the presence of either ch2A2 HuG2/G4 or ch3F4 HuG2/G4 antibodies. Finally, the chimeric antibodies were examined for the ability to block the adhesion of U-937 cells to purified soluble pVCAM (Fig. 11). Addition of the ch3F4 HuG4 antibody failed to inhibit binding of U-937 cells to pVCAM unless very high concentrations of antibody were added. In contrast the ch3F4 HuG2/G4 antibody potently inhibited the binding of U-937 cells to pVCAM, while the negative control h5G1.1 HuG2/G4 antibody did not block binding. Together, these results demonstrate the anti-pVCAM HuG2/G4 antibodies specifically inhibited the adhesive interaction of human VLA-4 with porcine VCAM and that disruption of this interaction was sufficient to inhibit binding of numerous human cell types to porcine endothelial cells.

DISCUSSION

Current success in allotransplantation has resulted from the development of effective global immunosuppressive therapies that prevent cellular graft rejection. Recent attempts to develop more specific immunosuppressive strategies have focused on inhibiting interactions between integrins and costimulatory molecules on T cells with their ligands on graft endothelial cells and other antigen presenting cells (reviewed in Faustman, 1995; Guinan *et al.*, 1994). The success of allotransplantation has been limited by the resulting shortage of human donor organs. Recent progress in overcoming the initial

Samsung Bioepis Exhibit No. 1002
Page 7574

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HuG2      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG2/G4   STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG4      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV

HuG2      HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG2/G4   HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG4      HTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVE

                                  235
HuG2      RKCCVECPPCPAPPV- AGPSVFLF
HuG2/G4   RKCCVECPPCPAPPV- AGPSVFLF
HuG4      SKYGPPCPSCPAPEFLGGPSVFLF

HuG2      PPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPR
HuG2/G4   PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR
HuG4      PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR

                                                      331
HuG2      EEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKG
HuG2/G4   EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
HuG4      EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
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Fig. 9. Sequence of the CH1, hinge and CH2 regions of human IgG2 (HuG2), human IgG4 (HuG4), and the chimeric HuG2/G4 construct. Positions where differences occur between the three molecules are double underlined. The amino acid sequence at position 235/236 determines ability to bind to Fcγ receptors (Canfield and Morrison, 1991; Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) while the presence of a serine at position 331 confers the inability to activate C (Tao *et al.*, 1993).

hyperacute rejection of discordant xenografts suggests the potential for use of porcine xenografts as a clinical option to circumvent this chronic shortage. Although there are limited data regarding the fate of discordant xenografts which survive the initial hyperacute rejection phase, xenograft recipients will likely require continued cellular immunosuppression for the prevention of graft rejection. In this light, xenografts offer a unique opportunity for the development of blocking reagents specific for the graft, thereby avoiding global immunosuppression of the recipient.

To investigate the interactions occurring between human leukocytes and porcine endothelial cells, we have cloned the porcine VCAM molecule and created mAbs specific for porcine VCAM that do not bind to human VCAM (Mueller *et al.*, 1995). Two of these antibodies (3F4 and 2A2) are capable of blocking the interaction between human VLA-4 and porcine VCAM. Here we demonstrate that the binding specificity for 2A2 is located within domain 1 of pVCAM while the binding specificity for 3F4 is located within domain 2. This is identical to the results found with human VCAM, where blocking antibodies have also been shown to be directed against either domains 1 or 2 (Osborn *et al.*, 1992; Vonderheide and Springer, 1992).

The use of murine mAbs in humans and primates presents numerous problems including a short antibody half-life and a high degree of immunogenicity (Khazaeli *et al.*, 1994). To optimize the use of the anti-porcine VCAM antibodies in porcine-to-primate xenograft experiments we developed chimeric antibodies using the variable regions cloned from the 2A2 and 3F4 antibodies joined to the constant regions of the human IgG4,κ isotype. Given the extensive activation of C which occurs within xenografts, the human IgG4 isotype was chosen due to its inability to activate C. The resultant chimeric antibodies (ch2A2 HuG4 and ch3F4 HuG4 antibodies) retained specificity for porcine VCAM and were as potent as the original murine mAbs at blocking adhesion of Ramos cells to activated PAEC by greater than 90% (Fig. 3). In contrast to Ramos, the binding of resting or activated human T cells to activated PAEC was inhibited only approximately 50% by either the chimeric antibodies or the original mAbs (Fig. 4). Activation of human T cells by PAEC can be blocked by antibodies directed against either CD2 or LFA-1 (Rollins *et al.*, 1994*b*), suggesting either of these molecules could mediate the remaining adhesion by interacting with porcine CD58 or porcine ICAM-1, respectively.

The adhesion of human lymphocytes, monocytes or NK cells to activated HUVEC is dependent on the interaction of both LFA-1 with ICAM-1 and VLA-4 with VCAM (Allavena *et al.*, 1991; Jonjic *et al.*, 1992; Vonderheide and Springer, 1992). For each cell type, blocking the VLA-4/VCAM interaction by itself produces only a small reduction in adhesion, while antibodies which disrupt the LFA-1/ICAM-1 interaction provide a greater, although still partial, inhibition of adhesion. Addition of antibodies blocking both VLA-4/VCAM and LFA-1/ICAM-1 is required to completely block adhesion of these cell types to HUVEC. Surprisingly, binding of LFA-1$^+$–VLA$^+$4 Jurkat or YT cells to activated PAEC could be inhibited nearly completely by the ch3F4 HuG4 and ch2A2 HuG4 antibodies. One possible explanation for this could be the lack of interaction between human LFA-1 and porcine ICAM. The conservation of ICAM-1 is relatively low between species, with human and mouse ICAM-1 being only 50% conserved at the amino acid level (reviewed in Carlos and Harlan, 1994), and *in vitro* mouse LFA-1 does not bind to human ICAM-1. Alternatively, human LFA-1 may interact with porcine ICAM-1, but with a reduced affinity. For example, human VLA-4 binds to human VCAM at two independent sites, one within domain 1 and one within

Samsung Bioepis Exhibit No. 1002
Page 7575

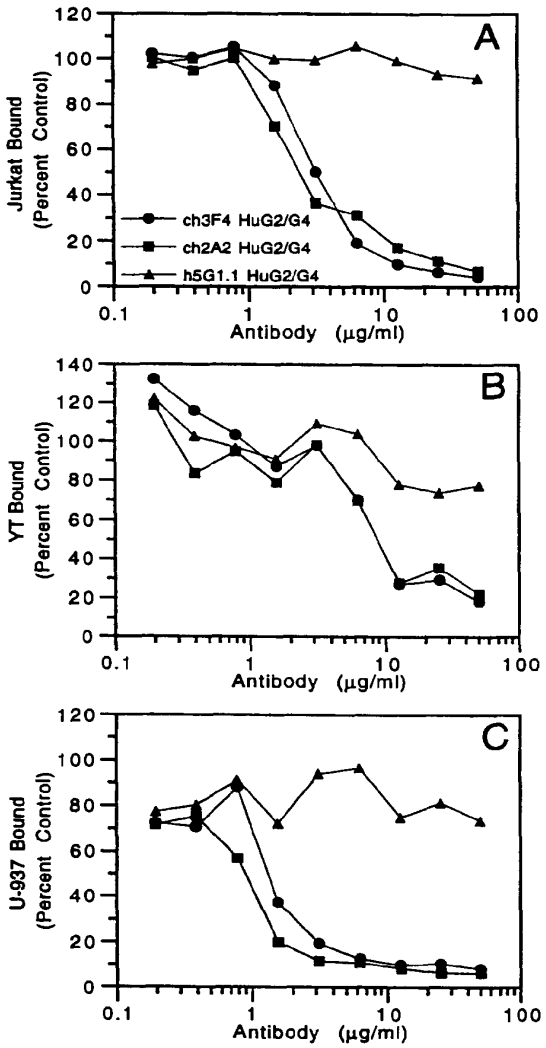


Fig. 10. Binding of Jurkat, YT and U-937 to PAEC in the presence of HuG2/G4 mAbs. Adherence of calcein-labeled Jurkat (A), YT (B) and U-937 (C) cells to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

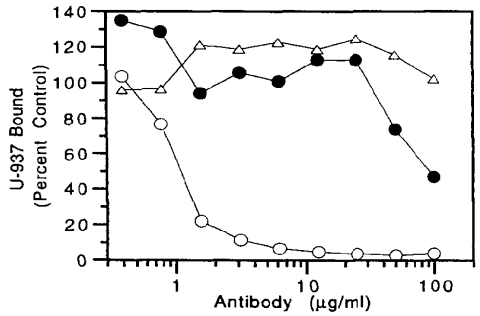


Fig. 11. Binding of U-937 to purified pVCAM. Microtiter plate wells were coated with purified soluble pVCAM. After blocking with FCS, wells received the indicated concentrations of either ch3F4 HuG4 (●), ch3F4 HuG2/G4 (○) or h5G1.1 HuG2/G4 (△) followed by 3×10^5 calcein-labeled U-937 cells. The cells were allowed to adhere at 37°C for 15 min after which non-adherent cells were removed by washing. Adherence of U-937 cells in the absence of competitor antibody was defined as 100%. Results shown are from an individual representative experiment.

domain 4 (Osborn *et al.*, 1992; Vonderheide *et al.*, 1994). In contrast, porcine VCAM lacks domain 4, suggesting the interaction between human VLA-4 and porcine VCAM may be weaker than the interaction between human VLA-4 and human VCAM. Reduced ligand affinities could make the binding of human effector cells to porcine endothelial cells more susceptible to disruption of any one of multiple weak interactions. These observations suggest the potential for significant differences in the therapeutic efficacy achieved by inhibiting particular receptor–ligand interactions with xenografts as compared to allografts.

Although the ch3F4 HuG4 and ch2A2HuG4 blocked binding of Jurkat and YT to activated PAEC, both antibodies failed to block binding of the monocytic U-937 cells to activated PAEC (Fig. 5). The levels of activated $\beta 1$ were comparable on Jurkat and U937 cells indicating an increased level of functional VLA-4 was not the mechanism behind the lack of inhibition of U-937 binding. Additionally, flow cytometry demonstrated the direct binding of the chimeric HuG4 antibodies to U-937 cells (Fig. 7). This binding was not dependent on the antigen specificity of the variable region, suggesting the interaction occurred through the Fc portion of the antibody. Finally, F(ab′)2 prepared from 3F4 HuG4 were able to block the adhesion of U-937 cells to activated PAEC by greater than 80% (Fig. 8). Together these results were consistent with a model in which the chimeric anti-pVCAM HuG4 mAbs crosslinked U-937 cells to activated PAEC by simultaneously binding FcRs and cell surface VCAM present on the PAEC. This is comparable to the situation encountered with the anti-CD3 murine monoclonal antibody (mAb) OKT3, which when first administered to patients produces crosslinking of T cells to FcR bearing cells, resulting in the activation of both cell types (Ceuppens *et al.*, 1985; Kan *et al.*, 1986) with resultant toxicity to the patient.

The interaction of antibodies with Fcγ receptors has been found to be dependent on the sequence of the hinge linker region of the CH2 domain (Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) as well as an additional bend located between two β strands within the CH2 domain (Canfield and Morrison, 1991). In the case of a humanized OKT3 antibody (human IgG4 constant regions), binding to Fcγ receptors has been eliminated by the introduction of amino acid mutations in the hinge linker (Alegre *et al.*, 1992, 1994). In either case the resulting human IgG4 constant region contains amino acid differences which could be immunogenic. Here, we have created chimeric antibodies (designated HuG2/G4) containing the CH1 domain and hinge linker region of

Samsung Bioepis Exhibit No. 1002
Page 7576

human IgG2 followed by the CH2 and CH3 domains of the human IgG4 constant region. Due to the similarities between these two isotypes at the amino acid level, the resultant antibody contained no novel peptides less than 30 amino acids in length likely to be presented by MHC class II molecules and invoke an immune response. The chimeric HuG2/G4 antibodies no longer bound to U-937 cells. Moreover, the ch2A2 HuG2/G4 and ch3F4 HuG2/G4 antibodies blocked the binding of Jurkat and YT cells to activated PAEC as potently as the chimeric HuG4 antibodies, demonstrating the alteration of the Fc region did not reduce the antibody avidities. Moreover, the chimeric anti-pVCAM HuG2/G4 recombinant antibodies nearly completely blocked the binding of U-937 cells to activated PAEC and to purified pVCAM. Finally, as predicted from the low affinity of IgG2 and IgG4 for human C1q, our preliminary results indicate that the HuG4 and HuG2/G4 recombinant antibodies show little or no C1q binding (data not shown).

In summary, we have developed humanized mAbs specific for porcine VCAM which potently block the adhesion of multiple human inflammatory cell types to porcine endothelial cells. The lack of binding of these antibodies to human VCAM leaves intact the interaction of human leukocytes with human endothelial cells. The adhesion-blocking properties of these antibodies will allow delineation of the role of VLA-4/VCAM interactions in cellular mediated rejection of porcine-to-primate xenografts. Additionally, the HuG2/G4 antibody design should prove useful in humanization of other antibodies intended for human use where elimination of FcR binding and C activation may be desirable.

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Samsung Bioepis Exhibit No. 1002
Page 7578

INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Q5C

Current *Step 4* version

dated 30 November 1995

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

Samsung Bioepis Exhibit No. 1002
Page 7579

Q5C
Document History

First Codification	History	Date	New Codification **November 2005**
Q5C	Approval by the Steering Committee under *Step 2* and release for public consultation.	29 March 1995	Q5C

Current *Step 4* version

Q5C	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	30 November 1995	Q5C

Samsung Bioepis Exhibit No. 1002
Page 7580

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Annex to the Tripartite ICH Guideline for the Stability Testing of New Drug Substances and Products

ICH Harmonised Tripartite Guideline

Having reached *Step 4* of the ICH Process at the ICH Steering Committee meeting on 30 November 1995, this guideline is recommended for adoption to the three regulatory parties to ICH

1. PREAMBLE

The guidance stated in the ICH harmonised tripartite guideline "Stability Testing of New Drug Substances and Products" (27 October 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products do have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products, in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. In order to ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With the above concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions which can affect the product's potency, purity and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

2. SCOPE OF THE ANNEX

The guidance stated in this annex applies to well-characterised proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using rDNA technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumour necrosis factors), erythropoietins, plasminogen activators, blood

Samsung Bioepis Exhibit No. 1002
Page 7581

plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterised proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

3. TERMINOLOGY

For the basic terms used in this annex the reader is referred to the "Glossary" in the ICH harmonised tripartite guideline "Stability Testing of New Drug Substances and Products" (27 October 1993). However, since manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

4. SELECTION OF BATCHES

4.1. Drug Substance (Bulk Material)

Where bulk material is to be stored after manufacture but prior to formulation and final manufacturing, stability data should be provided on at least 3 batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

4.2. Intermediates

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. While the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

Samsung Bioepis Exhibit No. 1002
Page 7582

4.3. Drug Product (Final Container Product)

Stability information should be provided on at least 3 batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating will be based upon the actual data submitted in support of the application. Since dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

4.4. Sample Selection

Where one product is distributed in batches differing in fill volume (e.g., 1 millilitre (ml), 2 ml, or 10 ml), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg) samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure and possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for 3 or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

5. STABILITY-INDICATING PROFILE

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasised in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to adequately demonstrate product stability.

5.1. Protocol

The dossier accompanying the application for marketing authorisation should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information which demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

5.2. Potency

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognised standard. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterised reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult since, in some cases, *in vitro* tests for biological activity and physicochemical characterisation are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product prior to conjugation/binding, assessing the release of the active compound from the second moiety, *in vivo* assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of *in vitro* testing.

Samsung Bioepis Exhibit No. 1002
Page 7584

5.3. Purity and Molecular Characterisation

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Due to the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical and immunochemical analytical methodologies should permit a comprehensive characterisation of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated and/or stress stability studies, consideration should be given to potential hazards and to the need for characterisation and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterised or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

5.4. Other Product Characteristics

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

> Visual appearance of the product (colour and opacity for solutions/suspensions; colour, texture and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilised cakes, pH, and moisture level of powders and lyophilised products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf-life.

Additives (e.g., stabilisers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to adversely affect the product and should be carefully evaluated (see below).

Samsung Bioepis Exhibit No. 1002
Page 7585

6. STORAGE CONDITIONS

6.1. Temperature

Since most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

6.2. Humidity

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

6.3. Accelerated and Stress Conditions

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information for future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information which may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. While the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

6.4. Light

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

6.5. Container/Closure

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampoules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period

Samsung Bioepis Exhibit No. 1002
Page 7586

specified in the instructions-for-use on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

6.6. Stability after Reconstitution of Freeze-Dried Product

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

7. TESTING FREQUENCY

The shelf-lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf-lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf-lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf-lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter.

For products with proposed shelf-lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the pre-approval or pre-licence stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol which supports elimination of specific test intervals (e.g., 9 month testing) for post-approval/post-licensure, long-term studies.

8. SPECIFICATIONS

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf-life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf-life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf-life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that clinical performance is not affected as discussed in the tripartite guideline on stability.

9. LABELLING

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate

Samsung Bioepis Exhibit No. 1002
Page 7587

freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

10. GLOSSARY

Conjugated Product

A conjugated product is made up of an active ingredient (for example, peptide, carbohydrate) bound covalently or noncovalently to a carrier (for example, protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

Degradation Product

A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products some degradation products may be active.

Impurity

Any component of the drug substance (bulk material) or drug product (final container product) which is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

Intermediate

For biotechnological/biological products, a material produced during a manufacturing process which is not the drug substance or the drug product but whose manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step prior to continuation of the manufacturing process. This includes material which may undergo further molecular modification or be held for an extended period of time prior to further processing.

Manufacturing Scale Production

Manufacture at the scale typically encountered in a facility intended for product production for marketing.

Pilot-Plant Scale

The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

Samsung Bioepis Exhibit No. 1002
Page 7588

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November 21, 2018

Opposition against European patent EP 2 359 834
(Application No. 11 001 632.6)
Title: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor: Alexion Pharmaceuticals, Inc.
Opponent: Amgen Inc.
Our Ref.: AMG17203OP

In preparation of Oral Proceedings and in Reply to Proprietor's Submission dated March 22, 2018

I. Requests

1. We maintain our request pursuant to Article 101(2) EPC that EP 2 359 834 (hereinafter "the opposed patent") be revoked in its entirety.

2. We further maintain our request for oral proceedings pursuant to Article 116(1) EPC, in case the Opposition Division does not consider the above request justified on the basis of our written submissions.

II. Newly submitted documents

3. We herewith submit the following documents in response to the Proprietor's Observations:

D39 Tacken, P.J. *et al. Blood* **2005**, *106(4)*, 1278-1285;

D40 WO 2004/050017 A2 (Bowdish, K. S. *et al.*, June 17, 2004);

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PR-Nr. 1278

Samsung Bioepis Exhibit No. 1002
Page 7589

D41 WO 2004/108158 A1 (Rother, R. P. *et al.*, December 16, 2004);

D42 U.S. Patent No. 6,407,213;

D43 Emery *et al.*, *Arthritis & Rheumatism* 2006, *54(5)*, 1390-1400;

D44 Rituximab 1997 Product Data Sheet;

D45 Declaration by Prof. Ronald Taylor, including CV (Appendix A); and

D46 Hillmen *et al.*, *N. Eng. J. Med.* 1995, 333:1253-8.

III. Inadmissible Amendments (Art. 100(c) EPC / Art. 76(1) EPC, 123(2) EPC) and Insufficiency of Disclosure (Art. 83 EPC)

4. We have noted the OD's preliminary views expressed under items 6 and 8 of the Preliminary Opinion according to which the requirements of Articles 76(1) and 83 EPC are met. We are grateful for the OD's guidance in this regard, although we respectfully disagree. We shall expand on the issue of compliance with the requirements of Articles 100(b) and (c) EPC in the course of oral proceedings.

IV. No Inventive Step (Article 100(a) EPC / Article 56 EPC)

5. As argued in our Notice of Opposition, **D1** may be regarded as the closest prior art, which was not disputed by the Proprietor. In the alternative, **D2** to **D4**, **D11** and **D18** all represent promising springboards for the claimed subject matter. The allegedly distinguishing features of the claimed subject matter over **D1** are

- the single-use dosage form of 300 mg,
- in a concentration of 10 mg eculizumab/mL
- resulting in a total volume of 30 mL
- wherein the solution is preservative free.

6. The application is silent about supposed technical effects of <u>any of these features</u>. As argued before (sections 36 to 42 of our Notice of Opposition), it is plainly obvious and clearly most convenient for the person of skill in the art to select an amount of 300 mg eculizumab in order to provide a composition that is suitable for the administration of individual doses of 600 mg or 900 mg, as disclosed *inter alia* in **D1** (e.g., [0082], [0088], [0091]). We also enclose a Declaration by Prof. Taylor (**D45**) which confirms this, referring to the example of the use of a single-use dosage form of rituximab of 500 mg for doses of 500 or 1000 mg to treat

rheumatoid arthritis (see **D43** and **D44**). We also note that a similar view was (rightly) taken by the Primary Examiner during prosecution of the opposed patent (Communication dated July 21, 2011, p. 3, 6th paragraph).

7. Further, based on obvious medical considerations, but also regulatory guidelines (**D19**, p. 1/9, 4th and 5th paragraph), the skilled person would avoid using preservatives in multi-dose preparations, whenever possible. Once an amount and concentration of active agent have been determined, the total volume is inherently defined. This leaves the antibody **concentration of 10 mg/mL** as the only possibly relevant distinguishing feature over the prior art.

8. Notably, and in agreement with our position, the Proprietor has focused on discussing the alleged technical effects of the concentration of 10 mg/mL.

9. On a side note, the Proprietor argues that PNH would require that patients start treatment very quickly after having been diagnosed (e.g., sections 4.2.2 and 4.2.3). This allegedly requires that eculizumab must be stored *"on site at a location nearby"* to be ready for use when a patient is identified. It is unclear why the specific *"nature of PNH"* would demand such storage nearby, though. In the absence of any tangible evidence that there is a difference in this regard between PHN and any other disease evoking significant symptoms, this remains a completely unsupported allegation which is contested.

10. Indeed, many of the documents cited in our Notice of Opposition or during prosecution contain general descriptions of PNH and the repercussions the disease has on a patient's life. None of the documents mentions that it would be necessary to start treatment immediately after diagnosis. It seems worth pointing out that PNH is a chronic condition and the treatment with eculizumab aims at improving the quality of life of afflicted patients (see, e.g., [0030] of **B1**). The therapy does not aim at curing an acutely life-threatening condition. Accordingly, trials described in the prior art commonly involved observation periods of up to 13 weeks after identification of eligible patients and before start of the treatment (cf. **D18**, p. 1234, right column 1st and 3rd full paragraph; but also [0070] of **B1**). This would hardly appear to be ethically justified if treatment would indeed have to start immediately, as purported by the Proprietor. We also note that **D18** reports the need to vaccinate PHN patients prior to treatment against *N. meningitides* (page 1234, right column, end of 2nd paragraph), again indicating that treatment was not initiated immediately upon diagnosis. Further, we refer to new reference **D46** (Hillmen, enclosed herewith), whose authors followed a group of 80 PHN patients (see Abstract) for up to 48 years and reported a median survival of 10 years (see the Discussion). None of the statement in this article indicates that PHN patients, once diagnosed, would need more immediate treatment than other chronically ill patients. The Proprietor's

allegations in this regard are thus not only unsupported but also contrary to the facts emanating from the documents on file.

11. Any requirements derived from this alleged need for immediate treatment (such as stability over 48 months) must therefore be rated as being artificially construed. The requirements a pharmaceutical composition suitable for use in the treatment of a patient suffering from PNH would have to meet are no different from the requirements pharmaceutical compositions for the treatment of other chronic diseases would have to satisfy.

IV.1 The concentration constitutes an arbitrary choice

12. We maintain that the concentration of 10 mg/mL, presented by the applicant as allegedly providing "the ideal balance" of properties (e.g. section 5.3.2, 5.3.10, 5.4.8, etc. of the submission), constitutes an arbitrary choice among numerous equally (or possibly even more) suitable alternatives.

13. It is established case law that a feature which is considered an arbitrary choice among equivalent variations cannot contribute to inventiveness (**T 206/91** (item 5.5) and **T 37/82** (item 3).

14. In this regard, we first of all note that the concept of "the *ideal balance of properties"* advanced by the Proprietor should be seen for what it is: a red herring which has been artificially created in the hope to successfully argue for an allegedly unobvious technical effect resulting from meeting said artificial (and wholly arbitrary) requirement. The suggestion under item 5.3.2 of the Observations that the allegedly "ideal" balance purportedly achieved by the claim features would have been instrumental to *"the approval of the claimed composition as a treatment for PNH"* has no basis whatsoever. There is no evidence that the regulatory bodies specifically preferred the "balance of properties" offered by the approved formulation over the "balance of properties" displayed by any other formulation. In fact, there is no such thing as an objectively "ideal balance" of properties of any pharmaceutical, since any "balancing" will be based on subjective elements and preferences.

15. As an aside, the approval obviously did not relate to *"the claimed composition"* but to an antibody that has been recombinantly produced in specific cells following a specific manufacturing and purification protocol, and which has been formulated in specific buffers and excipients, and at a specific pH. None of this is mentioned in the claim. There is no evidence that the "balance of properties" displayed by the approved product is shared by each and every formulation falling within the claim; rather, the contrary is to be assumed, as will be explained further below.

16. According to a more realistic proposal by the Proprietor, the underlying problem of the original filing lies in the provision of a(n alternative) useful treatment for patients suffering from PNH (section 5.10.2 of the submission). If anything, a pharmaceutical composition to serve this purpose must be suitable (or useful) for use in the treatment of patients suffering from PNH. Any requirement going beyond this (such as stability during storage over 48 months, maintaining over 98% monomer content, etc.) is – in the absence of any evidence to the contrary – arbitrary, and thus, cannot be invoked as justification for an inventive step.

17. With regard to the 98% monomer threshold we further note that according to **D20**, the standard of the World Health Organization (WHO) for commercial intravenous immunoglobulin products was an aggregate level of less than 5% (**D20**, p. 9, left column, 1st paragraph). This means that, according to the WHO, any antibody solution comprising at least 95% antibody monomer would be suitable for intravenous delivery and could thus be released into the market. It seems obvious that the Proprietor tries to generate an artificially high barrier to make an insignificant difference (if there is any) appear significant. The 98% monomer threshold is therefore not technically meaningful.

18. The Proprietor also failed to provide any evidence that would show that a monomer content of 98% was essential for maintaining biologic activity in an antibody formulation. As noted above there is no evidence that this exact threshold is technically relevant. Why would 96% or 95% monomer content be insufficient? Perhaps more importantly, there is no evidence of a correlation between aggregation and loss-of-activity for this specific antibody! This is even more relevant for the alleged requirement of stability for 48 months. It is apparent from the Proprietor's own allegations that Soliris® obtained market approval by the FDA with a shelf life of 24 months, as will be discussed in more detail below. This means that both, the Proprietor and the regulatory authorities considered a formulation with a shelf life of 24 months, when properly stored, to be sufficiently stable for use in the treatment of PNH.

19. In fact, the concentration of 10 mg/mL cannot serve to distinguish the claimed subject matter from the prior art for the purposes of Article 56 EPC, as there is no particular technical effect conferred by this specific concentration. This is *inter alia* evident from **D23** and **D25** and even from the Proprietor's own data.

20. **D23** presents data on solutions of eculizumab in the buffer which is used for the commercial formulations of the drug, using three different analytical methods, size-exclusion ultra high-performance liquid chromatography (SE-UHPLC), anion exchange high-performance liquid chromatography (AEX-HPLC), and reduced sodium dodecyl sulfate capillary electrophoresis (rCE-SDS). These methods allow for assessing both chemical and structural stability of an antibody solution.

21. The Proprietor has argued that the data presented with the declaration by Dr. Michael Treuheit may not be informative, as stability of the solution was tested *"only over a 13 week period"*, which would be significantly less than the 48-month stability allegedly demonstrated in **D33**. We note in this regard that the Proprietor (and his party expert Dr. Miano) has at the same time admitted that stability testing at elevated temperatures would provide a good indication of effects which might be seen at 2 to 8°C over a longer period of time (see section 5.3.5 of the submission). This is consistent with the statement of Dr. Treuheit (cf. section 4 of **D23**). The data presented in **D23** for elevated temperatures thus provides a good estimate of the behavior of the tested antibody solutions over a longer period of time than the tested 13 weeks.

22. The Proprietor finds the data presented in **D23** to be in line with the data submitted as part of the Miano declaration (**D33**), by selectively interpreting the data to suit his line of arguments. This selective and self-serving interpretation is, however, misleading.

23. Antibody degradation occurs via multiple pathways, some of which are interdependent, such as denaturation, aggregation, surface adsorption, chemical degradation (oxidation, deamidation, formation of acidic species), etc. This has been correctly noted by the Proprietor under section 4.3.3 of the submission. One particular degradation pathway does not necessarily dominate for any one antibody (see also section 4.3.4 of the submission), however, Dr. Miano has solely analyzed aggregation of the antibody at merely two different concentrations (cf. section 3 of **D33**). **D23**, in contrast, provides data on aggregation (SE-HPLC, rCE-SDS) and chemical degradation (AEX-HPLC, rCE-SDS) for five different concentrations. The rCE-SDS analysis, in particular, provides a good overview over different degradation pathways, as it allows for separation of both chemically and structurally altered species with very high resolution (cf. section 7 of **D23**).

24. A closer look at the data of **D23** reveals that all tested concentrations of eculizumab (i.e., 2, 5, 10, 30 and 50 mg/mL) were equally stable at 4°C over the tested period, as measured by SE-HPLC (**D23**, Appendix A). At elevated temperature (25°C), there is slight aggregation visible for 30 mg/mL and 50 mg/mL, but this is at levels that are hardly significant. The lower concentrations (10, 5 and 2 mg/mL) show levels of monomer at the latest time point that are higher than at the earliest time point. This may be taken as an indicator of experimental noise in the measurement and the observed decrease in the monomer content at 30 mg/mL and 50 mg/mL is well within these limits.

25. In any case, all concentrations show a monomer content that is above 98% at any time point and would thus be suitable for release into the market according to the (we believe arbitrary) limits defined by the Proprietor (section 3 of **D33** and e.g. sections 5.3.4, 5.3.6 of the submission) and even more so within the limit defined by the WHO (95% monomer). The

Proprietor alleged that the 30 mg/mL and 50 mg/mL concentrations would fall *"below the release parameter for commercial lots of eculizumab"* (section 5.4.5 of the submission), but this is clearly incorrect, as all tested solutions exhibit a monomer content that is comfortably above the 98% threshold at any time point.

26. In addition, the lower concentrations of 5 and 2 mg/mL exhibit a monomer content at the latest time point that is higher than for the 10 mg/mL solution. Thus, if one follows the interpretation of the Proprietor and takes these very small differences into account, 10 mg/mL is actually less stable than either 5 mg/mL or 2 mg/mL and thus obviously not the "ideal" concentration. If the observed increase of monomer content for the 5 mg/mL and 2 mg/mL is interpreted as noise, they are at least as stable as the 10 mg/mL solution, i.e., the concentration of 10 mg/mL is of no advantage.

27. We note at this point that the commercial formulation of eculizumab is diluted to a concentration of 5 mg/mL prior to administration to the patient (section 5.3.9 of the submission and **D24**, p. 2 under *"Preparation and Administration"*), which means that the higher concentration of 10 mg/mL is, in any case, of no benefit to the patient, when compared to 5 mg/mL. The infusion volume (and thus infusion time) would be identical no matter if the antibody was stored at a concentration of 10 mg/mL or 5 mg/mL (or alternatively 6, 7, 8 or 9 mg/mL).

28. When looking at the chemical degradation, as measured by AEX-HPLC in **D23** (Appendix B), there is, again, no significant difference between the tested concentrations. Slight degradation is visible for all samples at elevated temperatures, but no discernible advantage of any concentration is apparent. If anything, when taking minimal changes into account as the Proprietor did, the solution of 30 mg/mL shows slightly less chemical degradation at the latest time point (25°C), compared to 10 mg/mL. In addition, the AEX-HPLC analysis also makes clear that chemical degradation appears to be the more relevant degradation pathway for eculizumab, as all samples show markedly faster degradation than in the SE-HPLC assay.

29. The overall picture (chemical and physical degradation), as determined by rCE-SDS is in accordance with this analysis. If anything, the higher concentrations of 50 mg/mL and 30 mg/mL are slightly more stable than 10 mg/mL (purity at the latest time point) at 4°C. At 25°C some degradation takes place in all samples, but there is no difference between 10, 30 and 50 mg/mL. If anything, 5 mg/mL is slightly worse than the other concentrations.

30. In summary, **D23** shows that, leaving experimental noise aside, there is no difference in stability between solutions of eculizumab, formulated in the commercial buffer at concentrations of 2, 5, 10, 30 and 50 mg/mL. Even if the OD was inclined to take the minimal differences into consideration that are visible in this raw data, there is no clear picture. All

concentrations are well within the specifications purported by the Proprietor (at least 98% monomer content) and 10 mg/mL shows, if anything, slightly faster chemical degradation than 30 mg/mL. The concentration of 10 mg/mL provides no advantage, and hence provides no technical effect that may be taken into account for an assessment of inventive step.

31. According to the Proprietor, the Miano declaration (**D33**) shows the benefits of the concentration of 10 mg/mL. We note that **D33** greatly exaggerates entirely insignificant differences to make a minimal effect appear technically relevant (see in particular the scale on the y-axis). In addition, **D33** only analyzes two concentrations, 10 mg/mL and 30g/mL and therefore cannot serve as evidence that 10 mg/mL is in fact "ideal", or even just better than concentrations of, e.g., 25, 15, 9, 8, 5 mg/mL, etc. Two data points are obviously insufficient to draw such a conclusion.

32. As explained by the Proprietor, the initial shelf life of commercial formulations of eculizumab was determined to be 24 months, which was later extended to 30 months, when stored at 2°C to 8°C (see FDA letters annexed to **D33**). Any discussion relating to stability of the "bulk" drug prior to final formulation is immaterial, as no feature relating to repackaging of bulk product is recited in the claim as granted. This means that both the Proprietor and the regulatory agencies found a shelf life of 24 months to be sufficient for Soliris® to be approved and enter the market at the time the opposed patent was filed.

33. More importantly, as the current shelf life is specified to be 30 months, any final eculizumab drug product older than 30 months would have to be discarded. No responsible physician would administer eculizumab compositions older than the specified shelf life (**D24** at p. 6, under *"How Supplied/Storage and Handling"* explicitly states the obvious: *"Do not use beyond the expiration date stamped on the carton."*). It is thus entirely immaterial, if the composition claimed by the opposed patent is hypothetically stable for 36, 48, or even 100 months. Any stability beyond 24 months, the shelf life which Soliris® was initially approved with, or at most 30 months, makes no difference whatsoever in the context of the original disclosure.

34. Hence, any solution of eculizumab providing sufficient stability over 24 months, when stored properly, would be equally suitable for use in the treatment of patients suffering from PNH. The data provided in **D33** shows that both tested solutions provide this degree of stability (see Fig. 2 of **D33**). Even when the required shelf life is extended to 30 months, Fig. 2 of **D33** shows that both, 10 mg/mL and 30 mg/mL, exhibit a monomer content that is distinctly above 98% after 30 months of storage (and, of course above 95%). This means there is no technically relevant difference between the two concentrations, even if the arbitrary 98% threshold purported by the Proprietor was accepted as a measure.

35. If one assumes a correlation between antibody concentration and aggregation tendency (which the person of skill would do, as further discussed below), this would also be true for concentrations of, e.g., 25, 20 and 15 mg/mL and even more so for concentrations between 10 and 5 mg/mL. As noted above, Soliris® is diluted to 5 mg/mL prior to administration, so reducing the concentration to 5 mg/mL would not be detrimental to the patient in any way.

36. This means that both tested concentrations (and the hypothetical alternatives) are equally suitable for the provision of a solution of eculizumab for use in the treatment of PNH. Even if there were slight differences with regard to the formation of aggregates, they would be insignificant in the context of the original disclosure (and any technical problem retrospectively derived from it), which relates to methods of treatment for PNH.

37. Again we note as an aside that Dr. Miano solely analyzed aggregation and only by one particular analytical method. As explained above, aggregation is only one of numerous different degradation pathways that may affect the activity of an antibody drug. Concluding from this very limited data that 10 mg/mL would provide an "ideal" concentration, in particular with regard to biologic activity (sections 4.2.3, 4.3.1, 4.6.9 of the submission) is complete speculation. It is perfectly possible and, when taking **D23** into consideration, indeed not unlikely that chemical degradation at 10 mg/mL occurs at least as fast as at 30 mg/mL. In any case, biological activity was not tested by Dr. Miano and it is thus readily conceivable that the solutions lost their biologic activity without aggregating.

38. The Proprietor thus failed to submit any evidence that the claimed pharmaceutical composition actually provides any particular effect that is relevant in the context of providing a pharmaceutical composition for use in the treatment of PNH.

39. Lastly, **D25** represents further evidence that there is no actual difference in the stability of eculizumab when stored at concentrations between 10 mg/mL up to more than 100 mg/mL. **D25** is a patent application, filed by the Proprietor of the opposed patent and directed to high concentration formulations of anti-C5 antibodies, particularly eculizumab. The Examples of **D25** disclose a wealth of data regarding numerous criteria of antibody stability for eculizumab (**D25**, [0229]) solutions at concentrations of *inter alia* 10, 30 and 105 mg/mL (**D25**, [0244] to [0265]).

40. In section 5.4.6 of the submission, the Proprietor alleged that there was no indication of the relative stability of high concentration formulations compared to the claimed 10 mg/mL composition in **D25**. This is plainly incorrect. Solution V of **D25** is a solution of eculizumab in a buffer comprising 10 mM sodium phosphate, 150 mM sodium chloride and 0.02% polysorbate 80, at pH 7.0 (**D25**, [0262] to [0265]). This composition is identical to the commercial formulation of Soliris® (cf. section 2 of **D33**).

41. This solution is tested against four other solutions comprising eculizumab in concentrations of either 30 mg/mL or 105 mg/mL with regard to, e.g., appearance and osmolality, SDS-gel band pattern, biologic activity but also monomer content via SE-HPLC (**D25**, table 4). The analysis shows that all tested solutions exhibited a monomer content of at least 98% after storage for 24 months at 2 to 8°C, including 30 mg/mL in two different buffers (see also [0282] of **D25**). Thus, all tested solutions would comply with the requirements formulated by the Proprietor after storage for 2 years. There is no significant difference between any of the tested solutions with regard to monomer content. This is equally true with regard to biologic activity as is evident from Table 6 of **D25** (see also [0306]). The authors conclude that

> *"These results indicate that the formulations described herein maintain the functional as well as structural stability of the anti-C5 antibodies dissolved therein for at least 2 years at 2°C to 8°C."* (**D25**, [0306])

42. In summary, **D25** shows that eculizumab may be formulated at a range of different concentrations, particularly at 30 mg/mL and higher, with very good stability over at least 24 months. Moreover, **D25** shows that there are no meaningful differences in terms of stability and biologic activity between solutions at 10 mg/mL, 30 mg/mL and 105 mg/mL. With regard to stability of the antibody, any one of the solutions disclosed in **D25** would thus be equally suitable for being used in the treatment of patients suffering from PNH.

43. The Proprietor stated that the buffer was changed in **D25**, when moving from the stock solution of 10 mg/mL to the higher concentrations and the different solutions would therefore not be comparable (section 5.4.6 of the submission). Notably, the granted claim contains no limitation with regard to the buffer. This means that the purported technical effect ("ideal" stability) must be provided by the allegedly distinguishing feature (i.e., the concentration of 10 mg/mL) over the whole scope claimed (i.e., in any buffer, at any pH, etc.).

44. Further, if differences in the buffer would have a significant impact with regard to long term stability, which the Proprietor appears to suggest, it would be *prima facie* not credible that the technical problem (or technical effect) put forward by the Proprietor is solved (or achieved) over the whole scope claimed (again, this is also discussed in more detail below).

45. In the present case, the Proprietor explicitly relies on the concentration of 10 mg/mL as the sole feature allegedly providing the solution to the technical problem. The technical effect must therefore be provided by the concentration only and not depend on further features that are not recited in the claims (such as choice of buffer, excipients, pH, etc.). The solutions disclosed in **D25** are therefore perfectly comparable to the formulation as claimed, no matter which buffer was used. **D25** shows that the concentration of 10 mg/mL cannot serve as a

distinguishing feature, as it does not provide any technical effect over the other tested concentrations.

46. Notably, **D25**, which is specifically concerned with high concentration formulations of *inter alia* eculizumab and was filed by the Proprietor of the opposed patent, makes no note whatsoever of any challenges or unusual properties of the antibody arising from its allegedly unique and unusual structure. This sheds considerable doubt on the Proprietor's allegations in this regard.

47. In summary, the concentration of 10 mg/mL provides no relevant technical effect over other concentrations (such as 30 mg/mL), and therefore constitutes an arbitrary choice.

IV.2 The skilled person would arrive at the claimed solution without inventive activity

48. Even assuming a concentration of 10 mg/mL would provide any superior stability of eculizumab over other concentrations, which we contest, the skilled person would arrive at this concentration without inventive activity.

49. Starting from **D1**, the person of skill in the art would essentially be confronted with the problem of identifying a suitable concentration for eculizumab in a pharmaceutical composition for use in the treatment of PNH. Based on common general knowledge (such as **D20**, **D21**), the skilled artisan would be aware that antibodies are commonly formulated for intravenous administration at concentrations ranging from 1 mg/mL to 50 mg/mL (**D21**, table 1) or 0.5 mg/mL to 100 mg/mL (**D20**, table 1).

50. From **D22**, the skilled person would furthermore be aware that specifically eculizumab may be formulated at concentrations between 1 mg/mL and 30 mg/mL (**D22**, Fig. 10 and par. [0170] to [0172]). The Proprietor argued that the skilled artisan would not consider **D22**, as it relates to formulations for nebulization. While we agree that formulations for different routes of administration may be different in certain aspects, factors such as long term stability of the active substance and maintenance of biologic activity are clearly relevant for formulations for any route of administration. Thus, in the absence of any alternative guidance as to which concentration would be suitable for the specific antibody in question, the skilled person would take **D22** as an indication that eculizumab, formulated at a concentration of 1 to 30 mg/mL, would at the very least be sufficiently stable and biologically active to be used as a drug.

51. Importantly, even the buffers disclosed as being suitable in **D22** encompass the buffer used in the commercial formulation of eculizumab (Fig. 10, **D22**: 10 mM sodium phosphate, 150 mM sodium chloride, pH 6-8 and 0.001 to 0.02% Tween 80, compare section 2 of **D33**). "Tween 80" is synonymous for polysorbate 80 (see also [0069] of **D22**).

52. **D22** also discloses that eculizumab maintains its integrity after being formulated according to the teaching of **D22** and subsequent nebulization, as shown by SDS-PAGE and HPLC ([0173] of **D22**).

53. Thus, when faced with the task of identifying a concentration for eculizumab in a pharmaceutical composition for the treatment of PNH, the skilled person would not disregard **D22**, as it shows that eculizumab may be formulated at a concentration of 1 to 30 mg/mL while maintaining its structural integrity, i.e., without aggregating or precipitating.

54. The Proprietor tries to create the impression that **D22** would solely teach eculizumab formulations of 30 mg/mL and therefore teach away from the claimed subject matter (section 5.8.2 of the submission). This is evidently not the case. Fig. 10 of **D22** clearly shows that the concentration of the antibody may range from 1 mg/mL to 30 mg/mL, which obviously encompasses the claimed concentration of 10 mg/mL. Further, paragraphs [0171] and [0172] of **D22** teach that the *"Mab"* referred to in Fig. 10 is eculizumab.

55. Since eculizumab is a humanized monoclonal antibody with a hybrid IgG2/IgG4 constant domain, the skilled person would also readily refer to **D42** (entitled "Method for Making Humanized Antibodies"; submitted herewith) for guidance regarding concentrations. As noted by Prof. Taylor in **D45**, **D42** states that such humanized *"antibodies will typically be formulated … at concentrations of about 1 mg/ml to 10 mg/ml"* (**D42**, col. 45, li. 57, to col. 46, li. 2). **D42** relates to methods for making and using humanized antibodies, including antibodies with hybrid constant domains (**D42**, col. 8, ll. 47-51). Thus, selecting 10 mg/ml as concentration for a humanized mAb with a hybrid IgG2/IgG4 constant domain, such as eculizumab, would have been a typical, arbitrary choice among known concentrations.

56. We therefore submit that starting from **D1**, in combination with either **D22** or **D42** the skilled person would have had a reasonable expectation of success that a pharmaceutical composition comprising eculizumab in a concentration of 10 mg/mL would be useful in the treatment of PNH.

57. At the very least, the skilled person would have adopted a "try-and-see" attitude. We again refer to Board of Appeal decision **T 1241/03**. This case is indeed highly similar to the present one. The patent in suit *inter alia* related to *"aqueous liquid hGH formulations which are stable during long-term storage, namely 6 to 18 months at 2-8°C"* (**T 1241/03**, point 24 of the reasons, emphasis added). A document cited in the proceedings (document (7)) disclosed stable aqueous protein solutions, comprising *"growth and differentiation factors"* (point 28 of the reasons), i.e., not even the precise protein the patent in suit related to. Still, the competent Board found that

> *"[…] the skilled person, although knowing that the development of liquid stable protein formulations is technically unpredictable and does not allow to rely on stability predictions obtained from other, different samples, would not have been deterred from testing the stability of an hGH containing solution comprising the components indicated in document (7).*
>
> *Obviousness is not only at hand when the results are clearly predictable but also when there is a reasonable expectation of success (cf. decision T 149/93 of 23 March 1995; point (5.2) of the reasons). A reasonable expectation of success does not require certainty (cf. decision T 338/97, of 7 February 2000; point (14) of the reasons).*
>
> *Thus, in spite of the understandable uncertainties which always characterize experiments using biologic compounds like proteins, the skilled person had no reason to adopt a sceptical attitude. He/she would have had either some expectations of success or, at worst, no particular expectations of any sort, but only a "try and see" attitude, which - as pointed out in decisions T 333/97 of 5 October 2000; point (13) of the reasons - does not equate with the absence of an reasonable expectation of success (cf. decision T 1045/98 of 22 October 2001; point (17) of the reasons)."*
> (**T 1241/03**, point 31 of the reasons, emphasis added)

58. This approach has been endorsed in further Board of Appeal decisions, such as **T 1149/09** and **T 499/09**.

59. The Proprietor argued that formulation of antibody drugs was regarded *"at the time"* as being a highly complex and unpredictable issue. We note in this regard that **T 1241/03** was concerned with a patent that had a priority date in 1992, i.e., 15 years before the filing date of the opposed patent. Obviously, a task that was regarded to be within the scope of routine experimentation for a skilled artisan in 1992 would have been routine experimentation 15 years later.

60. The Proprietor has further argued that *"a "try and see" situation is considered by the Boards to be limited to particular situations, i.e. where the prior art clearly envisages a specific feature, and the skilled person merely has to determine by routine tests whether such feature does indeed provide a desired effect."* (section 5.5.4 of the submission, emphasis added). This is in fact exactly the case here. **D22** describes formulations of the same antibody (eculizumab) at concentrations ranging between 1 and 30 mg/mL, even in the same buffer. All the skilled person had to do was test a number of concentrations falling within the range disclosed in **D22** (in the very same buffers disclosed in **D22**) and determine their suitability, thereby immediately arriving at concentrations such as 10 mg/mL. Similar considerations apply to **D42**.

61. The Proprietor has stressed that it is essential to consider what the skilled person would have done instead of considering what the skilled person could have done (sections 5.5.1 and 5.5.2 of the submission). Indeed, the skilled person would have been aware of the inherently empirical nature of the task at hand, namely identifying (a) suitable concentration(s) for eculizumab. With this in mind, the skilled person would have looked for any prior art that would indicate a suitable starting point or springboard, which would have undoubtedly led to **D22**. The Proprietor argued that the skilled person would disregard **D22**, but if the alternative would be merely guessing a starting point for the empirical analysis it seems much more likely that the skilled person would have considered the teaching of **D22**, as it teaches stable pharmaceutical formulations of the same antibody. The Proprietor failed to provide any convincing argument why the skilled person would have disregarded **D22**. Further, the skilled person would have alternatively readily considered **D42** for the reasons explained above.

62. Thus, in view of the prior art, the skilled person would have envisaged a range of concentrations (1 to 30 mg/mL) and would subsequently have determined by routine testing which of said concentrations had the desired effect. When testing a limited number of concentrations in the relevant range, for example 1, 10, 20 and 30 mg/mL, and without modifying the teaching of **D22** in any way (i.e., in the same buffer) the skilled person would have instantly arrived at the claimed concentration of 10 mg/mL. No further modification would have been necessary. Alternatively, **D42** would have led the way to the claimed concentration.

63. The skilled person would have been further guided towards the alleged invention by his/her common general knowledge, particularly the known fact that aggregation propensity generally increases with concentration. The authors of review article **D20**, for example note that

> *"Increasing the concentration of antibodies often increases the aggregation tendency of the protein"* (**D20**, p. 9, left column, 2nd full paragraph).

While this differs from the Proprietor's interpretation of the disclosure of **D20** (cf. section 4.6.8 of the submission), the above quote from the document shows that the authors of **D20** would not share the Proprietor's view.

64. Thus, when testing concentrations within the limited range of 1 to 30 mg/mL disclosed in **D22**, the skilled person would have had a clear motivation and incentive based on the common general knowledge to not only test eculizumab at 30 mg/mL, but also at lower concentrations in the expectation of being able to reduce any aggregation tendency that might be found at 30 mg/mL.

65. Thus, the allegedly inventive feature of a concentration of 10 mg/mL would have been arrived at in an obvious manner by following routine experimentation.

66. The Proprietor tried to explain at some length why finding a suitable concentration for eculizumab was a tedious task, necessitating an inventive activity. First, the Proprietor suggested that the formulation of antibodies was a *"complex challenge"* (section 4.3 of the submission). In the absence of any features in the claim further defining the formulation we note that the only challenge the skilled person was faced with in the current case was the identification of suitable concentrations within the range of 1 to 30 mg/mL disclosed in **D22**, (or the range of 1 to 10 mg/ml disclosed in **D42**, for that matter) which would have been achieved (and the claimed solution thereby arrived at) by means of routine experimentation.

67. This is not changed by the fact that *"each antibody is unique"* (section 4.4 of the submission). Even if this was the case, it does not render finding a suitable antibody concentration inventive. Finding a suitable concentration belongs to the steps which are routinely undertaken when identifying a useful formulation for a specific antibody. According to established case law, work *"involving mere routine experiments, such as merely conventional trial-and-error experimentation without employing skills beyond common general knowledge, lack[s] inventive step (**T 455/91**, **T 104/92**)"* (Case Law of the Boards of Appeal, 8th Edition, section 9.18.5). It is not apparent which skills beyond the average and which insights going beyond mere common general knowledge would have been involved in finding suitable (or even the claimed allegedly *"ideal"*) concentration(s) for eculizumab.

68. Notably, many passages of the Proprietor's observations relate to the identification of, e.g., the right *"formulation components"* (section 4.4.1 of the submission), pH value (section 4.4.2 of the submission), *"pH and excipients"* (section 4.5.1 of the submission), lyoprotective additives (ibid.), and so on. Importantly, however, no features related to such parameters are recited in the granted claim. The Proprietor tries to draw a picture of the skilled person being confronted with a complex, multi-dimensional problem. This attempt must fail already in view of the lack of corresponding features of the claim of the opposed patent, and the lack of evidence of an ambitious technical problem having been solved. On the contrary, the skilled person would have been confronted with a rather straightforward task, namely the identification of suitable concentrations. Of course, the determination of suitable concentrations would have to be performed in any case, as the skilled person anyway would have to make a choice in this regard (and would be able to rely on simple and routine measures in this regard) when formulating an antibody.

69. With regard to antibody concentrations used in earlier formulations (section 4.6 of the submission, also section 47 of our Notice of Opposition), the Proprietor argued that the concentration of 10 mg/mL would not clearly emerge as the single most common concentration for pharmaceutical antibody formulations prior to the filing date of the opposed patent. This is, however, not necessary for a finding of obviousness. As noted above,

obviousness is not only at hand when the results are clearly predictable, but also when there is a reasonable expectation of success (**T 149/93**), which does not require certainty (**T 338/97**). This of course means that it is equally not necessary that a skilled person is able to predict a suitable formulation from his/her common general knowledge (section 4.6.7 of the submission). It is similarly immaterial that *"[t]here is no single technique or set of excipients and buffer/pH conditions, which formulation experts have at their disposal, which is guaranteed to lead to stability of any particular antibody product"* (section 4.6.8 of the submission, emphasis added).

70. As correctly summarized by the Proprietor, an observation that can be made based on the antibody products on the market before the filing date of the patent is that *"[t]hose products that can be stored in solution form include five products having a concentration from 1 to 2 mg/ml; four products having a concentration of 10 mg/ml; and three products having a concentration of 20 mg/ml or above"* (section 4.6.3 of the submission). Thus, even assuming 10 mg/mL was not the single most used concentration, it was undoubtedly a very common one. It is not apparent what would have deprived the skilled person of his/her reasonable expectation of success regarding the stability of eculizumab when being formulated at a concentration of 10 mg/mL, particularly not when taking the teaching of **D22** into consideration, which is specific for eculizumab.

71. Finally, the Proprietor puts heavy emphasis on the allegedly unique structure of eculizumab, which supposedly made finding a suitable concentration more challenging, compared to other antibodies. As noted above **D22** specifically discloses suitable concentrations for eculizumab and therefore any (anyway completely hypothetical and speculative) alleged problems due to the uniqueness of the antibody are irrelevant when taking **D22** as prior art into consideration. It further seems worth pointing out that the application as filed does not mention any challenges arising from the structure of eculizumab either. To the contrary, the original disclosure merely states that

> *"Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985)."* (**WO**, p. 22, li. 21/22)

The application as filed thus confirms that eculizumab may be formulated according to common procedures. It was also known prior to the filing date of the opposed patent that eculizumab comprises a chimeric IgG2/IgG4 constant region, as evidenced by **D39**, submitted herewith (**D39**, p. 1279, left column, under *"Recombinant antibodies"*). Contrary to the Proprietors allegation, there are other antibodies disclosed in the prior art which contain the same engineered heavy chain constant region (see **D15**, e.g., p. 8, point 9); **D16**, throughout

the document, e.g., p. 5, 10, 11, etc.; but also **D40**, SEQ ID 67 and **D41** p. 18, submitted herewith).

72. None of these documents mentions any complication arising from the structure of the heavy chain constant region, or teaches that the IgG2/IgG4-containing antibody would behave differently when formulating them from other antibodies. To the contrary, all of them contain statements that the disclosed antibodies may be formulated according to standard procedures (**D15**, p. 24, 3rd full paragraph; **D16**, p. 17/18; **D40**, paragraph bridging p. 36/37; **D41**, p. 16). As noted above, this is equally true for **D25**, which specifically relates to high concentration, long-term stable formulations of anti-C5 antibodies, such as eculizumab. Eculizumab was used in all examples of **D25**. Yet, **D25** makes no mention of any difference between eculizumab and other anti-C5 antibodies which would be due to its allegedly unusual heavy chain.

73. Notably, all of the above-mentioned patent applications **D15**, **D16**, **D25**, **D40** and **D41** were filed by Alexion, the Proprietor of the opposed patent.

74. Thus, based on the state of the art at the filing date of the opposed patent, there was nothing to suggest that eculizumab would require conditions for its formulation that would be significantly different from commonly used conditions. It is therefore not apparent that the skilled person would have been discouraged from testing commonly used concentrations and confirming the expected usefulness of such concentrations, including the concentration as claimed. Indeed, the concentration which according to the Proprietor is "ideal", can by no means be considered to be unusual.

75. In summary, the claimed subject matter is wholly obvious in view of **D1** in combination with **D22** (or **D42**) and/or common general knowledge.

IV.3 <u>The technical problem as formulated by the proprietor is not derivable from the application as filed and has in any event not been credibly solved</u>

76. When justifying the reformulation of the technical problem, the Proprietor pointed to the Guidelines of Examination (section 5.10.1 of the submission), which state that it may be possible to rely on new effects *"submitted subsequently during the proceedings by the applicant, provided that the skilled person would recognize these effects <u>as implied by or related to the technical problem initially suggested</u> (see G−VII, 11 and T 184/82)"* (emphasis added). Under section 5.10.3 of the submission, the Proprietor then recites the example at G-VII-11 of the Guidelines where pharmaceutical activity and toxicity are considered to be sufficiently related for the technical problem to be reformulated accordingly. However, the

opposed patent, and the application underlying it utterly fail to provide a sufficient basis for the reformulation of the technical problem attempted in the present case.

77. The problem initially suggested was the provision of a treatment for patients suffering from PNH using eculizumab:

> *"In certain aspects, the application provides a method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria by administering a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is eculizumab."* (**WO**, p. li. 24 to 28)

This is supported by various similar statements throughout the original disclosure. In accordance with this interpretation, the Proprietor formulated the underlying problem of the original filing to lie in the provision of a useful treatment for patients suffering from PNH (see also again section 5.10.2 of the submission).

78. The Proprietor then alleges that the application as filed would justify reformulating the technical problem as the *"provision of a composition of eculizumab which demonstrates the ideal balance of properties for use in the treatment of PNH, including enhanced stability and thus maintenance of biological integrity following prolonged storage in the absence of preservative, whilst also providing a low-volume liquid formulation having acceptable infusion times and which is convenient to store"* (section 5.11.1 of the submission).

79. We note that this is not only a wholly artificial but also a highly specific problem, and one that is simply not derivable from the application as filed. Even if, as in the example given in the Guidelines at G-VII-11, pharmaceutical activity and toxicity were to be considered sufficiently related to allow for changing the technical problem accordingly, this is not so in the case of pharmaceutical activity and, e.g., enhanced stability, maintenance of biological integrity following prolonged storage in the absence of preservative, low-volume liquid formulation having acceptable infusion times and convenience to store, **let alone the provision of the allegedly "ideal combination" of all of the above** (which would obviously require some comparison to the prior art). Rather, these are galenic questions. The opposed patent is, however, not concerned with galenic questions at all.

80. In this regard, we refer to the Case Law Book which notes at page 458 of the English version (under item II.E.1.14) that reformulating the technical problem is not in breach of Art. 123(2) EPC provided the problem as clarified and the solution proposed could have been deduced from the application as a whole. In this regard, the Case Law Book cites **T 871/08** which

rejected the reformulation of the technical problem vis-à-vis the prior art requested in that case as follows:

> *4.3.4 … The board observes that a reformulation of the problem taking into account the above arguments would only be allowable, if the new problem could be deduced from the application as filed when considered in the light of the closest state in the art (see in this respect T 0013/84, OJ EPO 1986, 253; T 0530/90, point 4.3 of the reasons). In the present case, there is no basis in the application as filed for the provision of the so-called "safety margin for stable operation" that the respondent emphasised during the oral proceedings, nor can the latter be deduced from the application as filed by a person skilled in the art.*
>
> *4.3.5 It follows that the problem is to be reformulated in less ambitious terms, namely, to provide an alternative process for the production of hydrogen peroxide according to the anthraquinone process."* (Emphasis added)

Similarly to the concept of the "*safety margin for stable operation"* which the proprietor in **T 871/08** creatively (but eventually unsuccessfully) invented, there is no basis in the application as filed in the light of the closest prior art for reformulating the technical problem to the convoluted construct proposed by the Proprietor.

81. Hence the Proprietor's artificial and arbitrarily defined "ideal balance of properties" must not be used as basis for the discussion of inventive step already for legal reasons.

82. In any case, the data comprised in the original filing, but also in **D33**, by no means show that the claimed composition provides an ideal balance of properties. It is not quite clear what properties the Proprietor has in mind when making this bold claim, but undoubtedly one property that would appear to be highly relevant for a patient suffering from PNH is the biologic activity / drug activity of the active agent (cf. e.g. sections 4.3.1, 4.6.9, 5.3.1, 5.3.3 of the submission). In section 5.3.3 of the submission, the Proprietor states that

> *"[i]t is, of course, critical that the antibody maintains its biological integrity and activity in the period between manufacture and use."* (emphasis added)

83. However, neither the application as filed, nor **D33** contain any information on the activity of the antibody in the tested solutions. No comprehensive data on biological integrity is provided, either. As the Proprietor stated (section 4.3.3 of the submission) and as further discussed below, biological integrity depends on numerous different degradation pathways one of which is aggregation. The mere fact that an antibody does not aggregate over a certain period of time is by no means sufficient to prove that it actually retained its activity, let alone that a

solution exhibits an *"ideal balance of properties"*. Not even biological integrity can be reliably evaluated by this single parameter.

84. According to **D20** (p. 8 to 14), the major degradation pathways for antibodies are physical degradation (i.e., denaturation and aggregation) and chemical degradation (i.e., disulfide bond formation/exchange, nonreducible cross-linking, deamidation, isomerization, oxidation, formation of acidic or basic species, C-terminal clipping, fragmentation and/or Maillard reaction). Of all these pathways, the Proprietor only analyzed one, namely aggregation.

85. **D21** notes in this regard that

> *"Probably the most important aspect of identifying a successful antibody drug formulation is in the identification of truly detrimental alterations rather than all of the modifications that might occur. Although, from a regulatory standpoint, it may be beneficial to understand all of the potential degradation events associated with an antibody formulation, it is the recognition of a stability-indicating parameter (or several parameters) that allows the formulation scientist to focus in on what is critical to development of a successful product. Here, a series of physico-chemical and bioanalytical assays can be used to identify these parameters [32]. Although in vivo bioassays may ultimately be required to validate a stability-indicating formulation parameter, the most critical tools at the disposal of the formulation scientist are rapid and reproducible assays to measure protein changes due to oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification that could lead to a loss of function of the antibody drug."* (**D21**, p. 690, right column, 1st full paragraph, emphasis added)

86. In the present case, the proprietor picked one out of many aspects and chose to present it as being indicative of an *"ideal balance of properties"*. This is not credible by any means when judged in view of the prior art, or in view of the Proprietor's own statements. In fact, it is not even clear if aggregation belongs to the *"truly detrimental alterations"* described in **D21**, or if it is of minor significance for the specific antibody in question, eculizumab.

87. To evaluate any *"balance of properties"*, it would obviously be necessary to evaluate more than one property. At the very least it would appear appropriate to determine *"oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification"* (**D21**, see above). One analysis that may be used to deliver a more complete picture is the rCE-SDS assay performed by Dr. Treuheit (**D23**, Annex C). It shows that there is no significant difference between the tested concentrations. If anything, it shows that 5 mg/mL is slightly less stable than the other tested samples. It does, however, not show any advantage in terms of overall stability (physical and chemical degradation) for the concentration of 10 mg/mL.

88. In summary, it is not credible from the application as filed, or the subsequently filed data (**D33**) that the technical problem as formulated by the Proprietor ("*provision of a composition of eculizumab which demonstrates the ideal balance of properties for use in the treatment of PNH, including enhanced stability and thus maintenance of biological integrity following prolonged storage […]*") has been solved. The original disclosure contains no data in this regard at all and **D33** does not in fact provide evidence for the alleged maintenance of biological integrity, but at best a minute effect, if any, on aggregation, which does not seem to be the most relevant variable for the maintenance of the biological integrity of eculizumab. Any assertion about "ideal" properties is mere speculation.

89. Hence, the technical problem put forward by the Proprietor cannot be derived from (or is insufficiently related to) to original disclosure and in any event has not been credibly solved. Thus, it cannot be accepted as basis for the evaluation of inventive step.

IV.4 The problem has in any event not been credibly solved over the whole scope claimed

90. Even if a concentration of 10 mg/mL was indeed accepted to provide any benefit over other concentrations in the specific formulation that was tested, and even if it were assumed that finding this solution would require an inventive effort (which it should not be for the above reasons), the technical problem would not be credibly solved over the whole scope claimed.

91. The Proprietor alleged in great detail that finding a suitable concentration for eculizumab was a highly challenging endeavor. While we disagree with this assessment, we note that if all of the Proprietor's arguments were indeed accepted as true, one would have to conclude that numerous relevant and in fact essential features are missing from the claim as granted.

92. The Proprietor argued that *"it is very important to maintain the integrity of the antibody and to minimize any possible degradation pathways"* (section 4.3.1 of the submission, emphasis added). Such degradation pathways would include *"denaturation, aggregation, surface adsorption, disulphide bond formation/exchange, non-reducible cross-linking, deamidation, isomerisation, oxidation, formation of acidic or basic species, C-terminal clipping, fragmentation and Maillard reaction. The skilled person looking to formulate an antibody therefore has a multitude of different problems to consider, each of which will apply in different ways to each individual antibody."* (Section 4.3.3 of the submission). As noted before, the data provided by the proprietor relates to only one of these pathways, namely aggregation.

93. The Proprietor further states that

> *"[…] the strategies available to minimize the various degradation pathways may conflict with one another. For instance, deamidation, a major degradation pathway in*

> *antibodies, has been shown to have a strong pH-dependence, with less deamidation occurring at pH 4 compared to pH 7 (**D20**, page 12, right-hand column penultimate paragraph). In contrast, antibody precipitation can be a significant problem at low pH values. **D20** reports (page 11, left-hand column first paragraph) that incubation of two mouse monoclonal antibodies (lgG2a and lgG 1) at 37° C for 32 days caused precipitation at pH 3 and pH 4 solutions for both antibodies. It is therefore difficult to create a stable antibody formulation due to the numerous degradation pathways possible, and the potentially conflicting strategies available to the formulation chemist for dealing with each of them in the context of each individual antibody."* (Section 4.3.5 of the submission)

94. Thus, if the Proprietor's statements are accepted, then optimization of the pH value of an antibody formulation is absolutely crucial to provide long term stability. Due to contrary tendencies with regard to different degradation pathways (deamidation and precipitation), such optimization is allegedly a challenging task. In any case, varying pH values will have a significant impact on the stability of the antibody in solution.

95. Another critical variable appears to be the buffer of the solution, according to the passage from **D21**, recited by the Proprietor in section 4.3.6:

> *"One approach is to find a pH where the minimal amount of degradation events such as deamidation and oxidation will occur. Another is to identify a buffer system that can stabilize the formulation at that pH without damaging the antibody."* (**D21**, p. 701, right hand column, emphasis added)

96. Thus, certain buffer systems may damage an antibody in solution, according to the Proprietor's submission, citing **D21**.

97. Further, specific salt concentrations may lead to dimerization of one particular antibody while not leading to aggregation of a closely related antibody (section 4.4.2 of the submission).

98. The fact that many stress factors may influence aggregation of an antibody during *inter alia* formulation and long term storage is summarized in **D34**, which was submitted by the Proprietor:

> *"Throughout these steps the antibody molecule may encounter several stress factors that can dramatically increase its propensity to aggregate (including variations of temperature, pH, protein concentrations, ionic strength, exposure to air-water interfaces and mechanical stress)."* (**D34**, p. 270, left column, 1st paragraph, emphasis added)

99. Thus, according to the Proprietor and the cited prior art, an individual antibody will be stable only at a specific concentration, at a specific pH, in a specific buffer with a specific amount of certain salts being present. In order to *"determine a suitable composition for the uniquely-engineered eculizumab antibody, the skilled person would have to conduct a significant research program to fully investigate the stability and biological integrity of the antibody, as well as the various factors (and their interplay) affecting each"* (section 5.5.5 of the submission, emphasis added).

100. However, of all the various factors, only the concentration is defined in the granted claim. Consequently, the alleged technical effect must solely be provided by the claimed concentration, at any pH, in any buffer, using any concentration of any salt, etc.

101. These parameters (including concentration) may in our view be determined by means of routine experimentation, particularly when starting from such a promising springboard as **D1** in combination with **D22** or **D42**. If, however, the OD was inclined to follow the Proprietor's argument that the identification of a suitable concentration requires inventive activity, this would equally apply to the other essential variables, such as buffer, pH and salt concentration of the antibody composition. In this case it would in fact appear that the vast majority of embodiments falling under the ambit of the claims could not possibly provide the claimed technical effect and thus could not be assumed to solve the problem, while at the same time the identification of functional embodiments would require an undue burden.

102. The Boards of Appeal have clearly decided, in particular in cases where the Applicant/Proprietor tries to reformulate the technical problem to be solved based on a subsequently invoked technical effect, that this can only be accepted if the problem was credibly/plausibly solved over the whole ambit of the claims. Further, the burden of proof in such a case lies with the Proprietor (see e.g. **T 1188/00**, point 4.5 of the reasons, **T 1417/05**, point 9.4 of the reasons, **T 1034/14**, point 5.4 of the reasons, **T 235/04** point 4.6 to 4.8 of the reasons). However, the Proprietor utterly failed to provide any evidence that the alleged technical effect is in fact provided over the whole scope of the claims.

103. As it is by no means credible/plausible that the technical effect of superior, ideal or merely suitable antibody stability over long periods of storage is provided by the concentration of 10 mg/mL over the whole range of conceivable pH values, buffers and salt concentrations, in particular not in light of the Proprietor's own arguments and the documents on file, the alleged technical effect must be disregarded for the assessment of inventive step (**T 668/94** point 8.3 of the reasons). Any other approach would be in stark contrast to the long standing principle that the extent of a patent monopoly, as defined by the claims, should reflect the technical contribution to the art (e.g. **T 939/92**, point 2.4.2 of the reasons, together with **T 409/91**, point 3.3 and 3.4 of the reasons).

104. The claim as granted lacks an inventive step at least because the technical problem has not been credibly / plausibly solved over the whole ambit of the claims, and for the reasons detailed above.

V. No Priority Entitlement (Article 87 EPC)

105. We have explained in our Notice of Opposition why the opposed patent is not entitled to the claimed priority of US 783070 P. We note that the Proprietor has not submitted any arguments regarding this issue. The filing date of the opposed patent, i.e. March 15, 2007 is thus considered to be the relevant date for any consideration of prior art.

VI. Summary

106. In view of all of the above, revocation of the patent in its entirety is fully justified.

Respectfully,

Dr. H. Ulrich Dörries
European Patent Attorney

UD/SI/KS

Enclosures
Documents **D39** to **D46**

RITUXAN™
Rituximab

DESCRIPTION

The RITUXAN (Rituximab) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. The antibody is an IgG_1 kappa immunoglobulin containing murine light- and heavy-chain variable region sequences and human constant region sequences. Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids (based on cDNA analysis) and has an approximate molecular weight of 145 kD. Rituximab has a binding affinity for the CD20 antigen of approximately 8.0 nM.

The chimeric anti-CD20 antibody is produced by mammalian cell (Chinese Hamster ovary) suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product. The anti-CD20 antibody is purified by affinity and ion exchange chromatography. The purification process includes specific viral inactivation and removal procedures.

RITUXAN is a sterile, clear, colorless, preservative-free liquid concentrate for intravenous (IV) administration. RITUXAN is supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials. The product is formulated for intravenous administration in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, and Sterile Water for Injection. The pH is adjusted to 6.5.

CLINICAL PHARMACOLOGY

General

Rituximab binds specifically to the antigen CD20 (human B-lymphocyte-restricted differentiation antigen, Bp35), a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes.[1,2] The antigen is also expressed on >90% of B-cell non-Hodgkin's lymphomas (NHL)[3] but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues.[4] CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation,[4] and possibly functions as a calcium ion channel.[5] CD20 is not shed from the cell surface and does not internalize upon antibody binding.[6] Free CD20 antigen is not found in the circulation.[2]

Pre-clinical Pharmacology and Toxicology

Mechanism of Action: The Fab domain of Rituximab binds to the CD20 antigen on B-lymphocytes and the Fc domain recruits immune effector functions to mediate B-cell lysis *in vitro*. Possible mechanisms of cell lysis include complement-dependent cytotoxicity (CDC)[7] and antibody-dependent cellular cytotoxicity (ADCC). The antibody has been shown to induce apoptosis in the DHL-4 human B-cell lymphoma line.[8]

Normal Tissue Cross-reactivity: Rituximab binding was observed on lymphoid cells in the thymus, the white pulp of the spleen, and a majority of B-lymphocytes in peripheral blood and lymph nodes. Little or no binding was observed in non-lymphoid tissues examined.

Human Pharmacokinetics/Pharmacodynamics

In patients given single doses at 10, 50, 100, 250 or 500 mg/m² as an IV infusion, serum levels and the half-life of Rituximab were proportional to dose. In 9 patients given 375 mg/m² as an IV infusion for four doses, the mean serum half-life was 59.8 hours (range 11.1 to 104.6 hours) after the first infusion and 174 hours (range 26 to 442 hours) after the fourth infusion. The wide range of half-lives may reflect the variable tumor burden among patients and the changes in CD20 positive (normal and malignant) B-cell populations upon repeated administrations.

Rituximab at a dose of 375 mg/m² was administered as an IV infusion at weekly intervals for four doses to 166 patients. The peak and trough serum levels of Rituximab were inversely correlated with baseline values for the number of circulating CD20 positive B cells and measures of disease burden. Median steady-state serum levels were higher for responders compared to nonresponders; however, no difference was found in the rate of elimination as measured by serum half-life. Serum levels were higher in patients with International Working Formulation (IWF) subtypes B, C, and D as compared to those with subtype A. Rituximab was detectable in the serum of patients three to six months after completion of treatment.

The pharmacokinetic profile of Rituximab when administered as six infusions of 375 mg/m² in combination with six cycles of CHOP chemotherapy was similar to that seen with Rituximab alone.

Administration of RITUXAN resulted in a rapid and sustained depletion of circulating and tissue-based B cells. Lymph node biopsies performed 14 days after therapy showed a decrease in the percentage of B-cells in seven of eight patients who had received single doses of Rituximab ≥100 mg/m². [9] Among the 166 patients in the pivotal study, circulating B-cells (measured as CD19+ cells) were depleted within the first three doses with sustained depletion for up to 6 to 9 months post-treatment in 83% of patients. One of the responding patients (1%), failed to show significant depletion of CD19+ cells after the third infusion of Rituximab as compared to 19% of the nonresponding patients. B-cell recovery began at approximately six months following completion of treatment. Median B-cell levels returned to normal by twelve months following completion of treatment.

There were sustained and statistically significant reductions in both IgM and IgG serum levels observed from 5 through 11 months following Rituximab administration. However, only 14% of patients had reductions in IgG and/or IgM serum levels, resulting in values below the normal range.

CLINICAL STUDIES

A multicenter, open-label, single-arm study was conducted in 166 patients with relapsed or refractory low-grade or follicular B-cell NHL who received 375 mg/m² of RITUXAN given as an IV infusion weekly for four doses. Patients with tumor masses >10 cm or with >5,000 lymphocytes/μL in the peripheral blood were excluded from the study. The overall response rate (ORR) was 48% (80/166) with a 6% (10/166) complete response (CR) and a 42% (70/166) partial response (PR) rate. Disease-related signs and symptoms (including B-symptoms) were present in 23% (39/166) of patients at study entry and resolved in 64% (25/39) of those patients. The median time to onset of response was 50 days and the median duration of response is projected to be 10 to 12 months.

In a multivariate analysis, the ORR was higher in patients with IWF B, C, and D histologic subtypes as compared to IWF A subtype (58% vs. 12%), higher in patients whose largest lesion was <5 cm vs. >7 cm in greatest diameter (55% vs. 38%), and higher in patients with chemosensitive relapse as compared to chemoresistant (defined as duration of response <3 months) relapse (53% vs. 36%). ORR in patients previously treated with autologous bone marrow transplant was 78% (18/23). The following factors were not associated with a lower response rate: age ≥ 60 years, extranodal disease, prior anthracycline therapy, and bone marrow involvement.

In a second multicenter, multiple-dose study, 37 patients with relapsed or refractory B-cell NHL received 375 mg/m² of RITUXAN as an IV infusion once weekly for four doses.[10,11] The ORR was 46% with a median duration of response of 8.6 months (range 2.6 to 26.2+). Single doses of up to 500 mg/m² were well-tolerated.[9]

Twenty patients have received two courses and one patient has received three courses of RITUXAN as 4 weekly infusions of 375 mg/m² per infusion. The percentage of patients reporting adverse events upon retreatment was similar to that reported following the first course, although the incidence of specific adverse events differed (see ADVERSE EVENTS). All patients had obtained an objective clinical response (CR or PR) to the first course of RITUXAN™ (Rituximab); upon retreatment, 6 of 12 patients evaluable for response obtained a complete or partial remission.

Twenty-nine patients with relapsed or refractory, bulky (single lesion of >10 cm in diameter), low grade NHL received 375 mg/m² of RITUXAN as four weekly infusions. The overall incidence of adverse events and the incidence of Grade 3 and 4 adverse events was higher in patients with bulky disease than in patients with non-bulky disease (see ADVERSE EVENTS). Ten of 21 patients evaluable for response have obtained a complete or partial remission.

INDICATIONS AND USAGE

RITUXAN is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma.

CONTRAINDICATIONS

RITUXAN is contraindicated in patients with known Type I hypersensitivity or anaphylactic reactions to murine proteins or to any component of this product. (See WARNINGS.)

WARNINGS

RITUXAN is associated with hypersensitivity reactions which may respond to adjustments in the infusion rate. Hypotension, bronchospasm, and angioedema have occurred in association with RITUXAN infusion as part of an infusion-related symptom complex. RITUXAN infusion should be interrupted for severe reactions and can be resumed at a 50% reduction in rate (e.g., from 100 mg/hr to 50 mg/hr) when symptoms have completely resolved. Treatment of these symptoms with diphenhydramine and acetaminophen is recommended; additional treatment with bronchodilators or IV saline may be indicated. In most cases, patients who have experienced non-life-threatening reactions have been able to complete the full course of therapy. (See DOSAGE and ADMINISTRATION.) Medications for the treatment of hypersensitivity reactions, e.g., epinephrine, antihistamines and corticosteroids should be available for immediate use in the event of a reaction during administration.

Infusions should be discontinued in the event of serious or life-threatening cardiac arrhythmias. Patients who develop clinically significant arrhythmias should undergo cardiac monitoring during and after subsequent infusions of RITUXAN. Patients with preexisting cardiac conditions including arrhythmias and angina have had recurrences of these events during RITUXAN therapy and should be monitored throughout the infusion and immediate post-infusion period.

PRECAUTIONS

Laboratory Monitoring: Complete blood counts (CBC) and platelet counts should be obtained at regular intervals during RITUXAN therapy and more frequently in patients who develop cytopenias (see ADVERSE EVENTS).

Drug/Laboratory Interactions: There have been no formal drug interaction studies performed with RITUXAN.

HAMA/HACA Formation: Human anti-murine antibody (HAMA) was not detected in 67 patients evaluated. Less than 1.0% (3/355) of patients evaluated for human anti-chimeric antibody (HACA) were positive. Patients who develop HAMA/HACA titers may have allergic or hypersensitivity reactions when treated with this or other murine or chimeric monoclonal antibodies.

Immunization: The safety of immunization with any vaccine, particularly live viral vaccines, following RITUXAN therapy has not been studied. The ability to generate a primary or anamnestic humoral response to any vaccine has also not been studied.

Carcinogenesis, Mutagenesis, Impairment of Fertility: No long-term animal studies have been performed to establish the carcinogenic or mutagenic potential of RITUXAN, or to determine its effects on fertility in males or females. Individuals of childbearing potential should use effective contraceptive methods during treatment and for up to 12 months following RITUXAN therapy.

Pregnancy Category C: Animal reproduction studies have not been conducted with RITUXAN. It is not known whether RITUXAN can cause fetal harm when administered to a pregnant woman or whether it can affect reproductive capacity. Human IgG is known to pass the placental barrier, and thus may potentially cause fetal B-cell depletion; therefore, RITUXAN should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether RITUXAN is excreted in human milk. Because human IgG is excreted in human milk and the potential for absorption and immunosuppression in the infant is unknown, women should be advised to discontinue nursing until circulating drug levels are no longer detectable. (See CLINICAL PHARMACOLOGY.)

Pediatric Use: The safety and effectiveness of RITUXAN in children have not been established.

ADVERSE REACTIONS

Safety data are based on 315 patients treated in five single-agent studies of RITUXAN. This includes patients with bulky disease (lesions >10 cm), those who have received more than one course of RITUXAN, and patients receiving 375 mg/m² for eight doses.

Infusion-Related Events: An infusion-related symptom complex consisting of fever and chills/rigors occurred in the majority of patients during the first RITUXAN infusion. Other frequent infusion-related symptoms included nausea, urticaria, fatigue, headache, pruritus, bronchospasm, dyspnea, sensation of tongue or throat swelling (angioedema), rhinitis, vomiting, hypotension, flushing, and pain at disease sites. These reactions generally occurred within 30 minutes to 2 hours of beginning the first infusion, and resolved with slowing or interruption of the RITUXAN infusion and with supportive care (IV saline, diphenhydramine, and acetaminophen). The incidence of infusion-related events decreased from 80% (7% Grade 3/4) during the first infusion to approximately 40% (5% to 10% Grade 3/4) with subsequent infusions. Mild to moderate hypotension requiring interruption of RITUXAN infusion with or without the administration of IV saline occurred in 32 (10%) patients. Isolated occurrences of severe reactions requiring epinephrine have been reported in patients receiving RITUXAN for other indications. Angioedema was reported in 41 (13%) patients and was serious in one patient. Bronchospasm occurred in 25 (8%) patients; one-quarter of these patients were treated with bronchodilators. A single report of bronchiolitis obliterans was noted.

Immunologic Events: RITUXAN induced B-cell depletion in 70 to 80% of patients and was associated with decreased serum immunoglobulins in a minority of patients. The incidence of infection does not appear to be increased. During the treatment period, 50 patients in the pivotal trial developed 68 infectious events; 6 (9%) were Grade 3 in severity and none were Grade 4 events. Of the

Samsung Bioepis Exhibit No. 1002
Page 7613

6 serious infectious events, none were associated with neutropenia. The serious bacterial events included sepsis due to Listeria (n=1), Staphylococcal bacteremia (n=1) and polymicrobial sepsis (n=1). In the post-treatment period (30 days to 11 months following the last dose), bacterial infections included sepsis (n=1); significant viral infections included herpes simplex infections (n=2) and herpes zoster (n=3).

Retreatment Events: Twenty-one patients have received more than one course of RITUXAN™ (Rituximab). The percentage of patients reporting any adverse event upon retreatment was similar to the percentage of patients reporting adverse events upon initial exposure. The following adverse events were reported more frequently in retreated subjects: asthenia, throat irritation, flushing, tachycardia, anorexia, leukopenia, thrombocytopenia, anemia, peripheral edema, dizziness, depression, respiratory symptoms, night sweats, and pruritus.

Hematologic Events: During the treatment period (up to 30 days following last dose) severe thrombocytopenia occurred in 1.3% of patients, severe neutropenia occurred in 1.9% of patients, and severe anemia occurred in 1.0% of patients. A single occurrence of transient aplastic anemia (pure red cell aplasia) and two occurrences of hemolytic anemia following RITUXAN therapy were reported.

Cardiac Events: Four patients developed arrhythmias during RITUXAN infusion. One of the four discontinued treatment because of ventricular tachycardia and supraventricular tachycardias. The other three patients experienced trigeminy (1) and irregular pulse (2) and did not require discontinuation of therapy. Angina was reported during infusion and myocardial infarction occurred 4 days post-infusion in one subject with a prior history of myocardial infarction.

Table 1.

Adverse Events ≥5% of Patients (N=315)

	Incidence All Grades	
	N	%
Any Adverse Event	**275**	**87**
Body As A Whole		
Fever	154	49
Chills	102	32
Asthenia	49	16
Headache	43	14
Throat Irritation	19	6
Abdominal Pain	18	6
Cardiovascular System		
Hypotension	32	10
Digestive System		
Nausea	55	18
Vomiting	23	7
Hemic and Lymphatic System		
Leukopenia	33	11
Thrombocytopenia	25	8
Neutropenia	21	7
Metabolic and Nutritional System		
Angioedema	41	13
Musculo-Skeletal System		
Myalgia	21	7
Nervous System		
Dizziness	23	7
Respiratory System		
Rhinitis	25	8
Bronchospasm	24	8
Skin and Appendages		
Pruritus	32	10
Rash	31	10
Urticaria	24	8

Severe and life-threatening (Grade 3 and 4) events were reported in 10% (32/315) of patients. The following Grade 3 and 4 adverse events were reported: neutropenia (1.9%), chills (1.6%), leukopenia and thrombocytopenia (1.3% for each), hypotension, anemia, bronchospasm, and urticaria (1.0% for each), headache, abdominal pain, arrhythmia (0.6% for each), and asthenia, hypertension, nausea, vomiting, coagulation disorder, angioedema, arthralgia, pain, rhinitis, increased cough, dyspnea, bronchiolitis obliterans, hypoxia, asthma, pruritus, and rash (one patient each, 0.3%).

The following adverse events occurred in ≥1.0% but <5.0% of patients, in order of decreasing incidence: flushing, artbralgia, diarrhea, anemia, cough increase, hypertension, lacrimation disorder, pain, hyperglycemia, back pain, peripheral edema, paresthesia, dyspepsia, chest pain, anorexia, anxiety, malaise, tachycardia, agitation, insomnia, sinusitis, conjunctivitis, abdominal enlargement, postural hypotension, LDH increase, hypocalcemia, hypesthesia, respiratory disorder, tumor pain, pain at injection site, bradycardia, hypertonia, nervousness, bronchitis, and taste perversion.

The proportion of patients reporting any adverse event was similar in patients with bulky disease and those with lesions <10 cm in diameter. However, the incidence of dizziness, neutropenia, thrombocytopenia, myalgia, anemia and chest pain was higher in patients with lesions >10 cm. The incidence of any Grade 3 and 4 event was higher (31% vs. 13%) and the incidence of Grade 3 or 4 neutropenia, anemia, hypotension, and dyspnea was also higher in patients with bulky disease compared with patients with lesions <10 cm.

OVERDOSAGE

There has been no experience with overdosage in human clinical trials. Single doses higher than 500 mg/m² have not been tested.

DOSAGE AND ADMINISTRATION

Usual Dose:

The recommended dosage of RITUXAN is 375 mg/m² given as an IV infusion once weekly for four doses (days 1, 8, 15, and 22). RITUXAN may be administered in an outpatient setting. **DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. (See Administration.)**

Instructions for Administration

Preparation for Administration: Use appropriate aseptic technique. Withdraw the necessary amount of RITUXAN and dilute to a final concentration of 1 to 4 mg/mL into an infusion bag containing either 0.9% Sodium Chloride USP or 5% Dextrose in Water USP. Gently invert the bag to mix the solution. Discard any unused portion left in the vial. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration.

RITUXAN solutions for infusion are stable at 2° to 8°C (36° to 46°F) for 24 hours and at room temperature for an additional 12 hours. No incompatibilities between RITUXAN and polyvinylchloride or polyethylene bags have been observed.

Administration: DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. Hypersensitivity reactions may occur (see WARNINGS). Premedication, consisting of acetaminophen and diphenhydramine, should be considered before each infusion of RITUXAN™ (Rituximab). Premedication may attenuate infusion-related events. Since transient hypotension may occur during RITUXAN infusion, consideration should be given to withholding antihypertensive medications 12 hours prior to RITUXAN infusion.

First Infusion: The RITUXAN solution for infusion should be administered intravenously at an initial rate of 50 mg/hr. RITUXAN should not be mixed or diluted with other drugs. If hypersensitivity or infusion-related events do not occur, escalate the infusion rate in 50 mg/hr increments every 30 minutes, to a maximum of 400 mg/hr. If hypersensitivity or an infusion-related event develops, the infusion should be temporarily slowed or interrupted (see WARNINGS). The infusion can continue at one-half the previous rate upon improvement of patient symptoms.

Subsequent Infusions: Subsequent RITUXAN infusions can be administered at an initial rate of 100 mg/hr, and increased by 100 mg/hr increments at 30-minute intervals, to a maximum of 400 mg/hr as tolerated.

Stability and Storage: RITUXAN vials are stable at 2° to 8°C (36° to 46°F). Do not use beyond expiration date stamped on carton. RITUXAN vials should be protected from direct sunlight.

HOW SUPPLIED

RITUXAN is supplied as 100 mg and 500 mg of sterile, preservative-free, single-use vials.
Single unit 100 mg carton: Contains one 10 mL vial of RITUXAN (10 mg/mL).
NDC 50242-051-21
Single unit 500 mg carton: Contains one 50 mL vial of RITUXAN (10 mg/mL).
NDC 50242-053-06

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Samsung Bioepis Exhibit No. 1002
Page 7614

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Declaration by Dr. Ronald Taylor

I, Ronald Taylor, Ph.D., declare that:

1. I was employed as a Professor of Biochemistry and Molecular Genetics at the University of Virginia from 1973 to 2016. I retired in 2016 and I am now Professor Emeritus and I still have an active work schedule based on submitting papers for publication, giving invited talks, serving on a study section, and reviewing papers. For almost 30 years, my laboratory has had a productive record in the study and characterization of monoclonal antibodies (mAbs), including humanized mAbs, and their use in clinical applications. A major focus of my research is anti-CD20 mAbs. In collaboration with investigators at the NIH and the Mayo Clinic, we have participated in numerous pilot clinical trials testing the feasibility of changing amount and frequency of dosing for CD20 mAb therapies for CLL. My work has resulted in 199 publications in peer-reviewed journals. A current copy of my CV is provided as **Appendix A.**

2. I have reviewed U.S. Patent Application Publication No. 2005/0191298 ("**D1**"), which is titled "Method of Treating Hemolytic Disease" and published on September 1, 2005. **D1** discloses that patients with paroxysmal nocturnal hemoglobinuria (PNH) were treated by administering eculizumab in four weekly 600 mg intravenous infusions, followed one week later by a dose of 900 mg, and then 900 mg on a bi-weekly basis (**D1**, [0082]). **D1** further discloses that anti-C5 mAbs such as eculizumab "can be administered in a variety of unit dosage forms" (**D1**, [0058]-[0060], [0062]).

3. The skilled artisan would have understood that pharmaceutical unit dosage forms containing a mAb should not be shared among patients or returned to storage after opening, in order to prevent contamination of the dosage form and any unnecessary health risks to the patient being treated with the dosage form. The skilled artisan also would have understood that

wasting mAb increases medical costs to the patient without any commensurate benefit. Thus, the skilled artisan would have had reason to prepare a single-use dosage form of eculizumab that could be used to administer both of the doses disclosed by **D1** (600 mg and 900 mg) without leaving any leftover drug as unusable waste. The skilled artisan would have understood that the most efficient way to meet that goal would be to prepare a 300 mg single-use dosage form because that dosage form could be used to administer both of the doses disclosed by **D1**—*i.e.*, two, 300 mg single-use dosage forms to administer the 600 mg dose and three, 300 mg single-use dosage forms to administer the 900 mg dose. Therefore, a 300 mg single-use dosage form would have been the most obvious and convenient single-use dosage form for preparing both the 600 mg and 900 mg doses of eculizumab for treating PNH.

4. The skilled artisan also would have recognized that preparing a single dosage form that could flexibly be used to administer either 600 mg or 900 mg offered advantages over developing a separate dosage form for each of the 600 mg and 900 mg doses. Developing any given dosage form requires testing, validating, manufacturing, storing, and transporting that dosage form. The skilled artisan would have known that it would be more efficient to avoid the redundancy and simply find a dosage form that could be used to administer both desired doses rather than creating multiple different dosage forms that would each require individual testing and validation, and may each require different storage or transport set-ups. Accordingly, efficiency would have provided the skilled artisan with further reason to use a 300 mg single-use dosage form.

5. One example of aligning antibody dosage form and administered doses known as of 2006 is the administration of the therapeutic antibody rituximab. Rituximab was administered in doses of 500 or 1000 mg to treat rheumatoid arthritis (e.g., Emery *et al.*, *Arthritis & Rheumatism* 2006, *54(5)*, 1390-1400 ("**D43**") at p. 1390). Consistent with maximizing efficiency and minimizing waste, the dosage forms of rituximab available as of 2006 were supplied as 100 mg and 500 mg sterile, preservative-free, single-use vials—both of which could be used to administer either a 500 or 1000 mg dose without waste and with maximal efficiency (e.g., Rituximab 1997 Product Data Sheet ("**D44**") at p. 2, right column, "How Supplied" section).

Samsung Bioepis Exhibit No. 1002
Page 7616

6. In preparing the 300 mg eculizumab single-use dosage form, the skilled artisan also would have considered the appropriate concentration at which to formulate the mAb. Eculizumab is a humanized mAb with a hybrid IgG2/IgG4 constant domain. U.S. Patent No. 6,407,213 ("**D42**") is titled "Method for Making Humanized Antibodies" and issued on June 18, 2002. **D42** relates to methods for making and using humanized antibodies, including antibodies with hybrid constant domains (**D42**, col. 8, ll. 47-51). **D42** states that such humanized "antibodies will typically be formulated … at concentrations of about 1 mg/ml to 10 mg/ml" (**D42**, col. 45, li. 57, to col. 46, li. 2). Thus, selection of 10 mg/ml as a concentration for a humanized mAb with a hybrid IgG2/IgG4 constant domain, such as eculizumab, would have been a typical, arbitrary choice among known concentrations. Similarly, U.S. Patent Application Publication No. 2005/0271660 ("**D22**") discloses using eculizumab at a concentration between 1 and 30 mg/ml in nebulized formulations (**D22**, Fig. 10, [0170]-[0172]). From this, the skilled artisan would have understood that eculizumab is sufficiently stable and biologically active to be used as a drug at concentrations including 10 mg/ml. Thus, **D22** reinforces that selection of 10 mg/ml as a concentration for eculizumab would have been a typical, arbitrary choice among known concentrations, regardless of the route of administration. **D44** reinforces the typical nature of a 10 mg/ml concentration because it discloses that rituximab was supplied at that concentration (e.g., **D44** at p. 2, right column, "How Supplied" section).

7. Additionally, in choosing among these *typical* concentrations, the skilled artisan would have had a reason to prepare the 300 mg single-use dosage form using a 10 mg/ml concentration of eculizumab in order to minimize the volume of the 600 mg and 900 mg doses required for treating PNH. For example, preparing the 600 mg and 900 mg doses taught by **D1** would require 60 ml and 90 ml, respectively, of a 300 mg single-use dosage form having 10 mg/ml of the mAb. But preparing the same 600 mg and 900 mg doses would require 600 ml and 900 ml, respectively, of a 300 mg single-use dosage form having 1 mg/ml of the antibody. The skilled artisan would have understood that smaller volumes would be more convenient for storage, transportation, and administration of the mAb. Further, the skilled artisan would have been reluctant to use concentrations higher than the 10 mg/ml taught by **D42** because increased aggregation was known to result from increased concentration of antibodies (e.g., Wang *et al*., *J. Pharm. Sci*. 2007, *96(1)*, 1-26 ("**D20**") at p. 9, left column, 2nd full paragraph).

Samsung Bioepis Exhibit No. 1002
Page 7617

8. Given that the skilled artisan would have had reason to use a 300 mg single-use dosage form with a concentration of 10 mg/mL, the skilled artisan necessarily would have arrived at a 30 mL volume. The volume of a 10 mg/mL solution needed to prepare 300 mg is, and can only be, 30 mL.

I certify that the above statements are correct to the best of my knowledge and belief.

Keswick, Virginia Nov 20, 2018
Place and Date

Dr. Ronald Taylor

Samsung Bioepis Exhibit No. 1002
Page 7618

Appendix A

Samsung Bioepis Exhibit No. 1002
Page 7619

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Ronald P. Taylor

eRA COMMONS USER NAME (credential, e.g., agency login): RPTNIH

POSITION TITLE: Emeritus Professor of Biochemistry and Molecular Genetics, University of Virginia Health Science Center

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
City College of New York, New York, NY	B.S.	1966	Chemistry
Princeton University, Princeton, NJ	Ph.D.	1970	Physical Chemistry
University of Minnesota, Mpls., MN	Post-doc	1971-1973	Physical Chemistry

A. Personal Statement

For almost 30 years my laboratory has had a long and productive record in the study and characterization of monoclonal antibodies and their use in several different clinical applications. We have a considerable level of experience in studying complement activation by, as well as effector cell-mediated killing of, tumor cells opsonized with monoclonal antibodies. Our laboratory discovered and has substantially characterized the shaving/trogocytosis reaction: that is, the loss of CD20 that occurs when CLL patients are treated with the usual (high) doses of rituximab or of ofatumumab. We have demonstrated, in several pilot clinical studies as well as in vitro investigations that much lower doses of CD20 mAbs, given more frequently, may provide far more effective therapies for CLL. We have also now described, in some detail at the cellular level, the complex series of events that occur when mAb-opsonized nucleated cells are killed by complement. Our laboratory also first described the exhaustion of complement which follows high dose rituximab therapy in CLL treatment.

I was appointed Professor Emeritus as of July 1, 2016. Since then I have maintained on-going collaborations with investigators in the US: Dr. D. Portilla (UVA), Dr. C. Zent (Rochester), Dr. A. Wiestner (NIH), Dr. J. Lambris (Penn), Dr. J. Thurman (U Colorado), Dr. S. Ram (U Mass); and in Europe: Dr. T. Mollnes (Norway) Dr. M. Huber-Lang (Germany), Dr. A. Verschoor (Germany) and the Genmab Biotech Company (The Netherlands).

B. Positions and Honors

Professional Positions:

1973-1978, Assistant Professor, Dept. of Biochemistry, University of Virginia.
1978-1983, Associate Professor, Dept. of Biochemistry, University of Virginia.
1983-present, Professor, Dept. of Biochemistry and Molecular Genetics, University of Virginia.
1991-present, Member, Beirne B. Carter Center for Immunology Research, University of Virginia.
1997-present, Member, NCI Sponsored Cancer Center, University of Virginia.

Awards and Other Professional Activities:

1965, Phi Beta Kappa; 1966, ECAC Scholar-Athlete Award; 1966-1969, NDEA Graduate Fellow; 1970, NSF Graduate Fellow; 1972-1973, NIH Fellow; 1975-1980, NIH Research Career Development Award; 1992, Ad

Samsung Bioepis Exhibit No. 1002
Page 7620

Hoc Study Section, Immunological Sciences, NIH; 1991-1993, Shannon Award, NIH; 1990-1994, Editorial Board, Arthritis and Rheumatism; 1990, Fellowship Subcommittee, Arthritis Fdn; 2008, DARPA Significant Technical Achievement Award; 2010-2015, Editorial Board, Haematologica; 2010-2014, Associate Editor, Journal of Immunology; Ad hoc reviewer: J. Immunology, J. Immunological Methods, J. Clin Invest, Blood, Mol. Immunology, Immunology, Science Translational Medicine, and many Cancer Journals: Reviewer, NIH: 2003-2004, Exptl. Immunol; 2004,Tumor Immunol./Immunotherapy; 2007-2018 (ongoing), Bench to Bedside NIH Study Section.

C. Contributions to Science (total publications: 199)

1. Our laboratory has pioneered the detailed and comprehensive evaluation of blood samples taken from patients before, during, and after CD20 therapy for CLL. This work led to our discovery of the shaving/trogocytosis reaction, as well as to the finding that immune effector functions in the body, such as complement, can be saturated/exhausted when CLL patients receive the usual high CD20 mAb doses (300 mg to 2 grams). This work also directly led to our in vitro analyses and mechanistic descriptions of trogocytosis, in which we demonstrated that it is mediated by Fcγ receptors on effector cells including monocyte/macrophages, neutrophils, and NK cells. We have also described the process in a mouse model and demonstrated that the reaction can be generalized to include other FDA-approved immunotherapeutic mAbs including cetuximab and traustuzumab. Finally, in collaboration with investigators at the NIH and the Mayo Clinic, we have been able to participate in numerous pilot clinical trials that have indicated the potential feasibility of changing CD20 mAb therapies for CLL to low doses (~35 mg) three times a week, which ultimately can be based on subcutaneous infusions. If this approach can be clearly validated and generalized, it would provide a very important and revolutionary paradigm shift that could benefit both patients and health-care providers, based on efficacy, costs, and convenience.

Most recently, in collaboration with several investigators we have also been studying how complement plays a role in the pathogenesis of several diseases and syndromes associated with inflammation and tissue damage, including malaria and kidney injury due to tubulointerstitial fibrosis. This work has the potential to lead to new and innovative strategies for disease treatment, based on selective inhibition of key steps in the complement cascade(s).

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Samsung Bioepis Exhibit No. 1002
Page 7621

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2. A second focus in our laboratory has been based on studying the basic scientific details by which mAbs promote CDC of targeted cells. This led to our discovery of "streamers," or tunneling nanotubules, that are released when the complement membrane attack complex penetrates the cell and large amounts of calcium enter the cytoplasm. We have also been able to describe the sequence of cellular events that occurs during the CDC process, based on 4-color movies generated in confocal microscopy experiments. This work, in collaboration with the biotech company Genmab, also led to our description of hexamer-forming mAbs that are

Samsung Bioepis Exhibit No. 1002
Page 7622

far more effective than wild-type mAbs in promoting CDC. It is very likely that these next generation mAbs (for CD20 as well as other targets) will find clinical applications in the not-too-distant future.

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Samsung Bioepis Exhibit No. 1002
Page 7623

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Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti–DC-SIGN antibody

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Current dendritic cell (DC)–based vaccines are based on ex vivo–generated autologous DCs loaded with antigen prior to readministration into patients. A more direct and less laborious strategy is to target antigens to DCs in vivo via specific surface receptors. Therefore, we developed a humanized antibody, hD1V1G2/G4 (hD1), directed against the C-type lectin DC-specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) to explore its capacity to serve as a target receptor for vaccination purposes. hD1 was cross-linked to a model antigen, keyhole limpet hemocyanin (KLH). We observed that the chimeric antibody-protein complex (hD1-KLH) bound specifically to DC-SIGN and was rapidly internalized and translocated to the lysosomal compartment. To determine the targeting efficiency of hD1-KLH, monocyte-derived DCs and peripheral blood lymphocytes (PBLs) were obtained from patients who had previously been vaccinated with KLH-pulsed DCs. Autologous DCs pulsed with hD1-KLH induced proliferation of patient PBLs at a 100-fold lower concentration than KLH-pulsed DCs. In addition, hD1-KLH–targeted DCs induced proliferation of naive T cells recognizing KLH epitopes in the context of major histocompatibility complex (MHC) classes I and II. We conclude that antibody-mediated targeting of antigen to DCs via DC-SIGN effectively induces antigen-specific naive as well as recall T-cell responses. This identifies DC-SIGN as a promising target molecule for DC-based vaccination strategies. (Blood. 2005;106:1278-1285)

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Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play a key role in regulating antigen-specific immunity. DCs capture antigens, process them into peptides, and present these to T cells.[1] The interaction between DC and T-cell controls the type and magnitude of the resulting immune response. Recently, preclinical and clinical studies have exploited DCs in an attempt to improve vaccine efficacy.[2] Most of these studies involve ex vivo antigen loading of autologous monocyte-derived DCs that are readministrated to the patient, a laborious and costly procedure. A more direct strategy involves targeting of antigens specifically to antigen uptake receptors on the DC in vivo. Potential candidate receptors highly expressed by DCs include Fc receptors[3-5] and members of the C-type lectin family.[6,7] Whereas Fc receptors are expressed by many different cell types, the expression of some members of the C-type lectin family are more DC restricted.[8]

C-type lectins bind sugar residues in a calcium-dependent manner via a highly conserved carbohydrate recognition domain. C-type lectin receptors expressed by DCs are implicated in immunoregulatory processes, such as antigen capture, DC trafficking, and DC–T-cell interactions.[8] Based on the location of the amino (N) terminus, 2 types of membrane-bound C-type lectins can be distinguished on DCs. Type I C-type lectins have their N terminus located outside, while type II C-type lectins have their N terminus located inside the cell. Several studies have been conducted on antigen targeting to C-type lectin receptors for vaccination purposes, mainly focusing on the type I C-type lectins mannose receptor (MR)[9] and DEC-205.[6,10,11] Vaccines based on natural MR ligands have been shown to effectively induce humoral and cellular responses.[9] However, these ligands lack specificity for the MR, and may target multiple lectins with overlapping binding specificities, including soluble lectins and lectin receptors expressed by cells that are not specialized in antigen presentation. More specific receptor targeting can be obtained by the use of antibodies directed against specific C-type lectins, a strategy that has been successfully applied in MR[7,12] and DEC-205[6,10,11] targeting studies. Antibody-mediated targeting of antigen to the MR on human DCs results in antigen presentation and activation of naive T cells in vitro.[7,12] Moreover, in vivo studies on antibody-mediated targeting of DEC-205 in mice demonstrate presentation of the antigen to naive $CD4^+$ and $CD8^+$ T cells.[6,10]

DC-specific intercellular adhesion molecule 3 (ICAM-3)–grabbing nonintegrin (DC-SIGN) represents a member of the

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Samsung Bioepis Exhibit No. 1002
Page 7624

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type II C-type lectin family. We have previously demonstrated that DC-SIGN is an endocytic receptor mediating antigen presentation.[13] A major advantage of targeting DC-SIGN over other C-type lectin receptors is its expression pattern. In humans, DC-SIGN expression is restricted to professional APCs and expression levels are high. Human DC-SIGN is abundantly expressed by DCs residing in lymphoid tissues and at mucosal surfaces, dermal DCs, and by specialized macrophages in placenta and lung.[14,15]

Targeting constructs that are to be used in humans should consist of antibodies that do not elicit immune responses directed against the antibody itself. Recent developments in antibody engineering provide the tools for the production of either humanized or human antibodies.[16] Undesired interactions between the targeting antibody and Fc receptors can be avoided by use of single-chain Fv constructs or composite immunoglobulin G (IgG) molecules,[17] thus enhancing targeting specificity.

Here we evaluate the effectiveness of targeting antigen to human DCs via DC-SIGN. For targeting purposes, keyhole limpet hemocyanin (KLH), a large globular protein containing a large array of immunogenic epitopes, was chosen as a model antigen. KLH is widely used in clinical DC-based vaccination trials for immunomonitoring purposes, and is thought to stimulate cytotoxic T-cell responses by recruiting bystander T-cell help.[2] KLH was chemically cross-linked to a humanized anti–DC-SIGN IgG2/IgG4 composite antibody (hD1), resulting in the chimeric hD1-KLH protein. The results demonstrate that hD1-KLH was capable of inducing T-cell responses at a 100-fold lower concentration than KLH alone.

Patients, materials, and methods

Antibodies and reagents

The following antibodies were used: AZN-D1 (IgG1, mouse antihuman DC-SIGN),[14] AZN-L19 (IgG1, mouse antihuman CD18),[18] W6/32 (IgG2a, mouse anti–HLA-A, –HLA-B, –HLA-C; ATCC, Manassas, VA), IVA-12 (IgG1, mouse anti–HLA-DR, –HLA-DP, –HLA-DQ; ATCC), mouse IgG1 isotype (R&D Systems, Abingdon, United Kingdom), total mouse IgG (Jackson ImmunoResearch; Brunschwig Chemie B.V., Amsterdam, the Netherlands), Alexa Fluor 647–labeled goat anti–human IgG (Molecular Probes, Leiden, the Netherlands), and Alexa Fluor 647–labeled goat anti–mouse IgG1 (Molecular Probes). Endotoxin-free KLH was purchased from Calbiochem (La Jolla, CA).

Recombinant antibodies

The humanized antihuman DC-SIGN antibody hD1V1G2/G4 (hD1) was generated by complementarity determining region (CDR) grafting of AZN-D1 hypervariable domains into human framework regions. The humanized variable heavy and variable light regions were then genetically fused with a human hybrid IgG2/IgG4 constant domain[17] and a human kappa chain constant domain, respectively. This construct was cloned into a mammalian expression vector and the final construct transfected into NSO cells. Stable transfectants were obtained using glutamate synthetase (GS) selection (Lonza Biologics, Portsmouth, NH). Supernatants containing hD1 were purified over a protein A column. An isotype control antibody, h5G1.1-mAb (5G1.1, eculizamab; Alexion Pharmaceuticals) containing the same IgG2/IgG4 constant region, is specific for the human terminal complement protein C5.[19]

Generation of hD1-KLH

The chemical cross-linker sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sSMCC; Pierce, Rockford, IL) was conjugated to KLH according to the manufacturer's protocol. Protected sulfhydryl groups were introduced to the hD1 antibody with N-succinimidyl-S-acetylthiopropionate (SATP; Pierce) and were reduced with hydroxylamine hydrochloride (Pierce) using the manufacturer's protocol. Subsequently, hD1 was added to sSMCC-treated KLH in phosphate-buffered saline (PBS, pH 7.4) and allowed to react for 16 hours at 4°C. Unbound sites were alkylated by adding iodoacetamide (Sigma-Aldrich, St Louis, MO) to a final concentration of 25 mM, followed by 30-minute incubation at room temperature. The protein mixture was loaded onto a Superose 6 column (24-mL bed volume; Amersham Pharmacia Biotech, Uppsala, Sweden), and fractions were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing hD1-KLH were pooled and fractions containing free hD1 were discarded. The efficiency of the cross-linking reaction was estimated by comparing the amount of hD1 relative to KLH before the reaction to the hD1–to–hD1-KLH ratio after cross-linking. We calculated that, on average, each KLH molecule had reacted with 10 hD1 molecules (data not shown). Endotoxin levels of the pooled hD1-KLH fractions were below detection levels (< 0.04 pg/μg protein) in the QCL-1000 *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Monocyte-derived DCs and PBLs

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy individuals and were purified using Ficoll density centrifugation. Peripheral blood lymphocytes (PBLs) and immature DCs (iDCs) were obtained from PBMCs as reported elsewhere.[14] In brief, PBMCs were allowed to adhere for one hour at 37°C. Nonadherent cells (PBLs) were gently removed, washed, and cryopreserved. The adherent monocytes were cultured in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 500 and 800 U/mL, respectively; Schering-Plough International, Kenilworth, NJ) for 6 days to obtain immature DCs. Mature DCs (mDCs) were obtained by culturing iDCs in the presence of 2 μg/mL lipopolysaccharide (LPS) for 24 hours. DCs were cryopreserved until use. Unless indicated otherwise, cells were cultured in X-VIVO 15 medium (Cambrex, Verviers, Belgium) supplemented with 2% human serum.

Tritiated thymidine incorporation assays

Tritiated thymidine (1 μCi [0.037 MBq]/well; MP Biomedicals, Amsterdam, the Netherlands) was added to the cell cultures. Tritiated thymidine incorporation was measured after 16 hours in a β-scintillation counter. Proliferation indices higher than 2 were considered positive.

Clinical vaccination protocol

PBLs and monocyte-derived DCs were isolated from melanoma patients participating in a clinical vaccination trial, as described by de Vries et al.[20] Patients were determined to have stage IV disease according to the American Joint Committee on Cancer (AJCC) criteria.[21] The study was approved by the institutional review board (Radboud University Nijmegen Medical Centre, Commissie Mensgebonden Onderzoek). Informed consent was provided according to the Declaration of Helsinki. The vaccination protocol consisted of 2 parts. In the first part, antigen-pulsed mDCs were administered intravenously and intradermally, 3 times at biweekly intervals. In the second part, patients received 3 monthly intradermal vaccinations with peptides alone (100 μg) and KLH (2 μg). Patients who remained free of disease progression after the first vaccination cycle were eligible for maintenance cycles at 6-month intervals, each consisting of 3 biweekly intranodal vaccinations in a clinically tumor-free lymph node region under ultrasound guidance with mDCs alternately pulsed with wild-type or modified glycoprotein 100 (gp100) peptides,[20] tyrosinase peptides, and KLH. Clinical grade DCs for vaccination purposes were generated from PBMCs as described previously.[22]

Humoral responses to KLH

Humoral responses to KLH were determined by enzyme-linked immunosorbent assays (ELISA) as described by Holtl et al.[23] Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 μg/mL) in PBS (0.1

Samsung Bioepis Exhibit No. 1002
Page 7625

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mL/well). Subsequently, plates were incubated with serial dilutions of patient serum, obtained before and during the third maintenance cycle, for one hour at room temperature. After extensive washing, human IgG-specific antibody labeled with horseradish peroxidase was allowed to bind for one hour at room temperature. Peroxidase activity was revealed using 3,3′ 5,5′ tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm. A signal detected at 1:400 or higher dilution of serum was considered positive.

Cellular responses to KLH

Cryopreserved PBMCs, isolated from peripheral blood samples taken from the patients, were thawed, washed, and plated at 1×10^5 PBMCs per well of a 96-well tissue culture microplate either in the presence or absence of 10 μg/mL KLH. After 4 days of culture, a tritiated thymidine incorporation assay was performed.

Binding and internalization assays

Binding of hD1 and hD1-KLH to iDCs was assessed by immunofluorescence and flow cytometry. iDCs were incubated with or without 10 μg/mL hD1 or hD1-KLH. In some experiments, DC-SIGN was blocked by pretreating iDCs with 100 μg/mL AZN-D1. After a one-hour incubation at 4°C, cells were washed and incubated with Alexa Fluor 647–labeled anti–human IgG antibody. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

Internalization of hD1 by iDCs was determined by flow cytometry as described previously.[24] Briefly, iDCs were incubated with 10 μg/mL hD1, AZN-D1, AZN-L19, or mouse IgG1 and 5G1.1 isotype control antibodies at 4°C for 30 minutes, washed, and incubated for 0, 15, 30, or 45 minutes at 37°C. Subsequently, some of the cells were fixed, while others were fixed and permeabilized in PBS/0.1% (vol/wt) saponin (Sigma-Aldrich) before addition of the Alexa Fluor 647–labeled anti–human IgG secondary antibody. The amount of internalized antibody was calculated by subtracting the mean fluorescence in fixed cells (surface bound) from that recorded with fixed and permeabilized cells (internalized and surface bound) at the various time points.

Internalization of hD1 and hD1-KLH was confirmed by confocal laser scanning microscopy (CLSM). iDCs were incubated with 10 μg/mL hD1, hD1-KLH, AZN-D1, or isotype control 5G1.1 and mouse IgG1 antibodies for one hour at 37°C. Cells were fixed on poly-L-lysine–coated glass slides, followed by intracellular staining with Alexa Fluor 647–labeled secondary antibodies. Cells were imaged with a Bio-Rad MRC 1024 confocal system operating on a Nikon Optiphot microscope and a Nikon × 60 planApo 1.4 oil immersion lens (Bio-Rad, Hercules, CA). Pictures were analyzed with Bio-Rad Lasersharp 2000 and Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) software.

KLH binding and internalization by iDCs was assessed by direct labeling of KLH using the Alexa Fluor 488 labeling kit (Molecular Probes). iDCs were incubated with 10 μg/mL labeled KLH for one hour at either 4°C or 37°C. Subsequently, cells were washed and analyzed by flow cytometry.

Live imaging of hD1-KLH and KLH uptake by iDCs

iDCs were labeled with LysoTracker Red (Molecular Probes) in PBS for 10 minutes at room temperature. Subsequently, cells were transferred to RPMI 1640 without phenol red (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 1% human serum. Labeled cells were analyzed at 37°C with a Zeiss LSM 510 microscope equipped with a type S heated stage CO_2 controller and PlanApochromatic 63 × 1.4 oil immersion DIC lens (Carl Zeiss, Jena, Germany). KLH and hD1-KLH, directly labeled with the Alexa Fluor 488 labeling kit (Molecular Probes), were added to the medium at 10 μg/mL. Cells were imaged using Zeiss LSM Image Browser version 3.2 (Carl Zeiss) and processed with Image J version 1.32j software (National Institutes of Health, http://rsb.info.nih.gov/ij).

Targeting experiments

iDCs were incubated with hD1, hD1-KLH, or KLH for one hour at 4°C or 37°C. Where indicated, iDCs were matured with LPS. Subsequently, DCs were washed and cocultured with KLH-responsive PBLs (ratio 1:10) at 37°C. In some experiments, iDCs were matured with LPS before addition of PBLs. After 16 hours of coculture, IL-2, IL-4, IL-5, IL-10, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) were measured by cytometric bead array (CBA) (T helper 1 [Th1]/Th2 Cytokine CBA 1; BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. After 4 days of coculture, a tritiated thymidine incorporation assay was performed.

Presentation of KLH epitopes over time

iDCs were incubated with 5 μg/mL KLH or 5 μg/mL hD1-KLH for one hour at 37°C. Following washing, KLH-responsive PBLs were added to the iDCs either on the same day, or 2 or 4 days later. Four days after addition of the PBLs, proliferative responses were determined in a tritiated thymidine incorporation assay.

Activation and expansion of naive T cells

Experiments were performed essentially as described previously.[7] Briefly, PBLs from a healthy donor were used as a source of T cells. Autologous DCs were incubated with 10 μg/mL hD1-KLH for one hour at 37°C, washed, and matured. Targeted mDCs were incubated with PBLs (ratio, 1:10) in the presence of IL-7 (10 ng/mL, day 0), followed by addition of IL-10 (10 ng/mL) on day 1 and IL-2 (20 U/mL) on day 2. IL-2 was added to the culture every 3 to 4 days. PBLs were restimulated each week. Restimulations were performed with iDCs treated with 5 μg/mL hD1-KLH after 1 week, and with iDCs treated with 2.5 μg/mL hD1-KLH after 2, 3, and 4 weeks. Finally, PBLs were harvested and cocultured with autologous mDCs that had been pulsed with KLH, as described in "Targeting experiments." Proliferative responses were determined by tritiated thymidine incorporation assays.

Results

Humanized anti–DC-SIGN antibody hD1 is internalized by iDCs

The CDRs of the mouse antihuman DC-SIGN antibody AZN-D1 were grafted onto a human IgG2/IgG4 composite antibody to generate a humanized antibody for DC-SIGN targeting, hD1. We have previously shown that the human hybrid IgG2/IgG4 constant domain prevents antibodies from binding to Fc receptors.[17] The binding affinity of hD1 for DC-SIGN was similar to that of AZN-D1 as determined by surface plasmon resonance (3.7 ± 0.7 nM and 3.8 ± 1.1 nM, respectively, data not shown).

Flow cytometric analysis revealed specific binding of hD1 to DC-SIGN on iDCs, as preincubation of iDCs with AZN-D1 efficiently reduced binding (Figure 1A). Furthermore, hD1 bound to DC-SIGN–expressing K562 cells after transfection with DC-SIGN cDNA, whereas it did not bind to untransfected K562 cells (data not shown). A time-course internalization experiment revealed that both the hD1 and the AZN-D1 antibodies were rapidly internalized by iDCs, although the AZN-D1 antibody was internalized slightly more efficiently than hD1. As expected, the control AZN-L19 antibody, directed against CD18, was not internalized (Figure 1B). Analysis by confocal microscopy confirmed internalization of the hD1 and AZN-D1 antibodies by iDCs, whereas the control antibodies 5G1.1, directed against human terminal complement protein C5, and mouse IgG1 isotype were not internalized (Figure 1C).

hD1-KLH is rapidly internalized and translocated to the lysosmal compartment

To further explore the potential of DC-SIGN as a targeting receptor for DC vaccination, we studied the uptake of KLH complexed to

Samsung Bioepis Exhibit No. 1002
Page 7626

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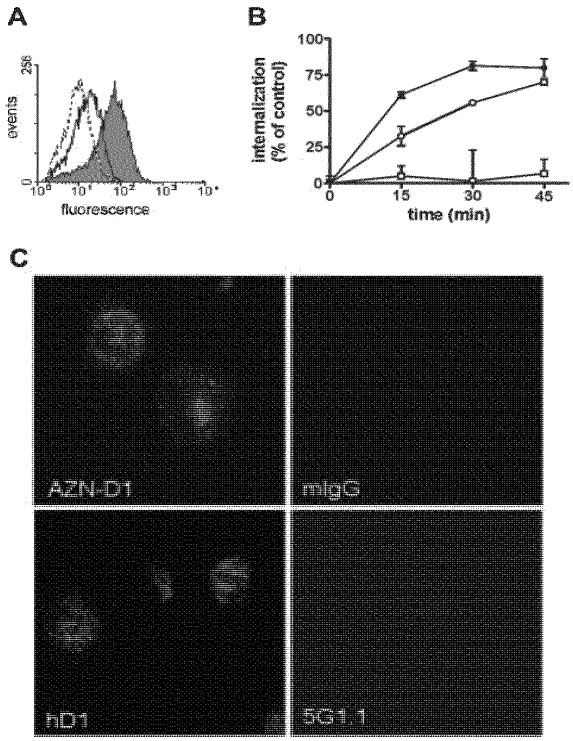


Figure 1. Binding of hD1 to DC-SIGN and internalization by DCs. (A) iDCs were treated with 10 μg/mL hD1 (gray shaded curve), 100 μg/mL AZN-D1 (open dotted curve), or pretreated with 100 μg/mL AZN-D1 followed by 10 μg/mL hD1 incubation (open solid curve), followed by incubation with an Alexa Fluor 647–labeled goat anti–human IgG antibody. Binding of hD1 was analyzed by flow cytometry. (B) iDCs were incubated with AZN-D1 (●), hD1 (○), or AZN-L19 (□) at 4°C for one hour, and transferred to 37°C. Cells were fixed at various time points, and stained with Alexa Fluor–labeled secondary antibodies with or without prior permeabilization. The mean fluorescence was determined by flow cytometric analysis, and the amount of internalized antibody was plotted as a percentage of the amount of total cell-associated antibody. Data represent experiments performed in triplicate ± SD. (C) Internalization of hD1 was confirmed by CSLM. iDCs were incubated with hD1, AZN-D1, or their isotype controls 5G1.1 and mouse IgG1 (mIgG) for one hour at 37°C. Cells were stained with Alexa Fluor 647–labeled secondary antibodies (blue), followed by microscopic analysis. The image represents the middle focal plane of the DCs, with iris set at 2 nm. Original magnification, ×600.

the humanized anti–DC-SIGN antibody hD1. As was observed with the hD1 antibody, hD1-KLH bound to iDCs (Figure 2A). hD1-KLH binding to DCs is mediated by DC-SIGN, as binding could be blocked by pretreating iDCs with AZN-D1 (Figure 2A). Analysis by CLSM revealed that hD1-KLH was rapidly internalized by iDCs (Figure 2B). After one hour of incubation at 37°C, hD1-KLH could be detected in 74% of the iDCs (data not shown). In contrast to hD1-KLH, KLH itself did not bind to iDCs, but was internalized by iDCs following one hour of incubation at 37°C (Figure 2C). These findings demonstrate that internalization of KLH by DCs is not receptor mediated, and likely depends on macropinocytosis.

Foreign antigens are taken up by DCs and shuttled, via endosomes, to the lysosomal compartment where they can be processed. In order to establish whether hD1-KLH reaches the lysosomal compartment, and to study uptake-kinetics and routing of KLH and D1-KLH, these proteins were directly labeled with Alexa Fluor 488 dye. Live imaging by CLSM revealed rapid uptake of hD1-KLH and KLH by iDCs. Both hD1-KLH and KLH were detected in the lysosomal compartment within one hour after their addition to the culture medium (Figure 2D; Supplemental Videos S1 and S2; see the Supplemental Videos link at the top of the online article, at the *Blood* website).

Induction of KLH responses by DC vaccination

In order to compare the antigen-presentation capacity of DCs pulsed with KLH to that of DCs targeted by hD1-KLH, we required KLH-specific T cells. To this end, PBLs were isolated from melanoma patients participating in a vaccination study. This study involved vaccination of patients with autologous DCs pulsed ex vivo with KLH and melanoma-associated antigens. Monocyte-derived DCs and PBLs from patients showing humoral as well as cellular responses against KLH were used for the targeting studies.

All 31 vaccinated patients participating in the clinical vaccination trial showed responses against KLH (data not shown). Humoral responses were readily detected in the patients' serum. KLH-specific IgGs were present after the first vaccination cycle, and their serum levels increased upon the second vaccination (Figure 3A). Cellular responses were analyzed using PBMCs obtained from patients one week before the start of the third vaccination cycle. Typically, PBMCs pulsed with KLH showed an increased proliferative response compared with unpulsed PBMCs, revealing the presence of KLH-reactive T cells in peripheral blood

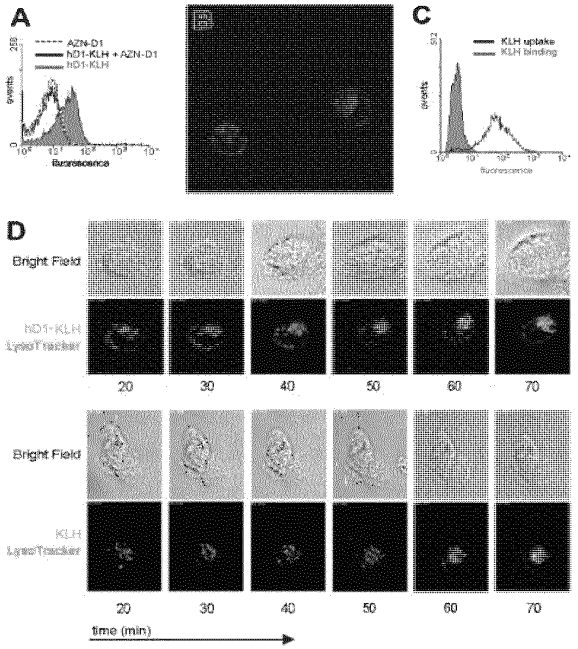


Figure 2. Binding and uptake of hD1-KLH by DCs. (A) iDCs were treated with 10 μg/mL hD1-KLH, 100 μg/mL AZN-D1, or pretreated with 100 μg/mL AZN-D1 followed by 10 μg/mL hD1 incubation, followed by incubation with an Alexa Fluor 647–labeled goat anti–human IgG antibody. Binding of hD1-KLH was analyzed by flow cytometry. (B) Internalization of hD1-KLH was confirmed by CSLM. iDCs were incubated with Alexa Fluor 647–labeled hD1-KLH (blue) for one hour at 37°C, followed by microscopic analysis. The image represents the middle focal plane of the DCs, with iris set at 2 nm. Original magnification, ×600. (C) iDCs were incubated with Alexa Fluor 488–labeled KLH for one hour at 4°C or 37°C. Subsequently, cells were fixed and analyzed by flow cytometric analysis. (D) iDCs were labeled with LysoTracker Red, followed by addition of Alexa Fluor 488–labeled (green) hD1-KLH or KLH. Cells were imaged by CSLM. Data represent bright field and corresponding fluorescent images of cells at various time points after addition of hD1-KLH or KLH to the culture medium. Original magnification, ×630.

Samsung Bioepis Exhibit No. 1002
Page 7627

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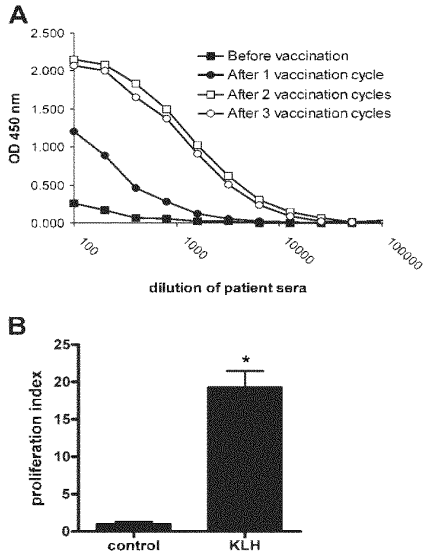


Figure 3. Humoral and cellular responses against KLH in patients vaccinated with KLH-pulsed DCs. (A) Patient serum was obtained both before the first, and after the first, second, and third vaccination cycles. Total IgG antibodies specific for KLH were detected by ELISA. Data represent optical density (OD) 450 values of serially diluted serum samples for a representative patient. (B) Patient PBMCs were isolated one week prior to the third vaccination cycle and cultured in the absence (control) or presence of 10 μg/mL KLH (KLH). Cellular responses were assessed in a tritiated thymidine incorporation assay. Data represent mean ± SD of experiments performed in 6-fold for a representative patient. Significant difference from control according to Student *t* test: $^*P < .001$.

(Figure 3B). These findings show that KLH-pulsed mDCs effectively induced both humoral and cellular responses in vivo.

Targeting of KLH to DC-SIGN results in presentation of KLH antigen epitopes

To demonstrate that antibody-mediated targeting of antigen to DC-SIGN leads to antigen presentation, autologous iDCs were incubated with 5 μg/mL KLH (± 0.63 nM) or hD1-KLH (± 0.53 nM) at 4°C for one hour, washed to remove unbound protein, and added to PBLs. Presentation of KLH epitopes to specific T cells was determined by measuring proliferative responses (Figure 4A-B) and the levels of cytokines secreted in the medium (Figure 4C). As endocytotic processes are inhibited at 4°C, and KLH does not bind to iDCs (Figure 2C), hD1-KLH can be internalized only after binding to DC-SIGN. iDCs incubated with hD1-KLH induced cellular responses by the PBLs, whereas iDCs incubated with KLH did not (Figure 4A-C), showing that targeting of KLH to DC-SIGN results in antigen presentation. We have previously demonstrated that the clinical vaccination protocol used in our studies results in a Th1-type immune response.[20] Indeed, stimulation of patient PBLs with hD1-KLH–treated iDCs resulted in enhanced secretion of IL-2, IFN-γ, and TNF-α (Figure 4C), while IL-4, IL-5, and IL-10 levels were not detectable (data not shown). As expected, iDCs incubated with KLH at 37°C induced proliferative responses, as did iDCs incubated with hD1-KLH (Figure 4A).

Presentation of KLH epitopes by iDCs incubated with hD1-KLH at 4°C was abolished by preincubation of iDCs with the hD1 antibody, but was unaffected by preincubation with the isotype control 5G1.1 (Figure 4B-C). This confirms that hD1-KLH targeted specifically to DC-SIGN, resulting in presentation of KLH epitopes. Cross-linking of hD1 to KLH was required for induction of DC-SIGN–mediated presentation of KLH epitopes, since iDCs incubated with both KLH and hD1 antibody did not induce cellular responses (Figure 4B-C).

Targeting KLH to DC-SIGN enhances its immunogenicity

To determine whether DC-SIGN targeting increases the efficiency of antigen presentation, immature monocyte-derived DCs from previously immunized patients were incubated at 37°C with various concentrations of KLH or hD1-KLH. Subsequently, iDCs were washed, and a portion of the iDCs was matured with LPS. hD1-KLH by itself did not induce maturation of iDCs (data not shown). Both hD1-KLH–treated iDCs (Figure 5A) and mDCs (Figure 5B) induced cellular responses at 100-fold lower concentrations than KLH-pulsed DCs. Thus, antigen targeting via DC-SIGN significantly enhances immunogenicity.

KLH epitopes are presented up to 4 days after targeting DC-SIGN

After antigen uptake in the periphery, DCs migrate to the draining lymph nodes to present the processed antigen to T cells. It is

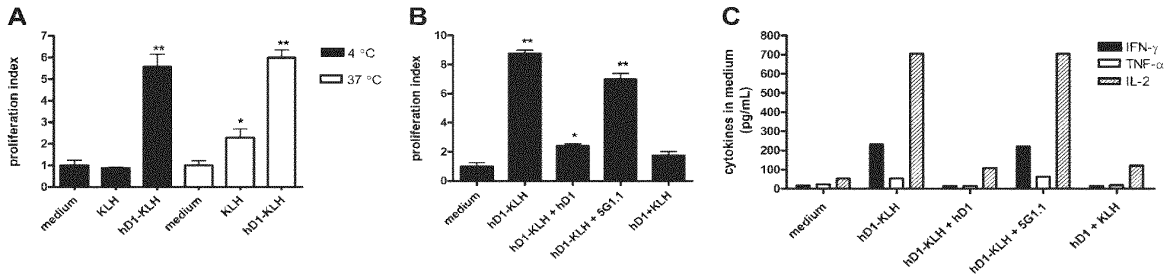


Figure 4. Antibody-mediated targeting of antigen to DC-SIGN results in antigen presentation. (A) iDCs were incubated with medium (control), 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 4°C or 37°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from the patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to analysis of variance (ANOVA) and Bonferroni test: $^*P < .05$; $^{**}P < .001$. (B) iDCs were incubated with medium (control), 5 μg/mL hD1-KLH, 5 μg/mL hD1-KLH and 100 μg/mL hD1 (hD1-KLH + hD1), 5 μg/mL hD1-KLH and 100 μg/mL 5G1.1 (hD1-KLH + 5G1.1), or 5 μg/mL hD1 and 5 μg/mL KLH (hD1 + KLH) for one hour at 4°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to ANOVA and Dunnett test: $^*P < .05$; $^{**}P < .01$. (C) Production of IFN-γ, TNF-α, and IL-2 in the coculture experiment described in panel B. After 16 hours of coculturing iDCs and PBLs, supernatants were taken and cytokine levels were determined. Data represent cytokine levels in pooled samples of experiments performed in triplicate.

Samsung Bioepis Exhibit No. 1002
Page 7628

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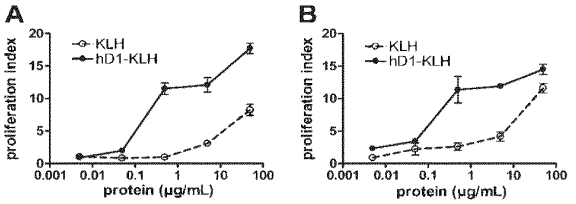


Figure 5. Targeting of antigen to DC-SIGN enhances its immunogenicity. (A) iDCs were incubated with medium, 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 37°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. (B) Experiment performed as described in panel A, except that antigen-treated iDCs were matured with LPS before addition of PBLs.

imperative that DCs present the antigens over a prolonged period of time to initiate a significant immune response. We evaluated the efficiency of antigen presentation over time following targeting by incubating patient PBLs with autologous iDCs that had been pulsed with KLH or hD1-KLH 0, 2, or 4 days earlier. The results demonstrate that DCs targeted with hD1-KLH presented KLH epitopes up to 4 days after targeting. Moreover, at all time points evaluated, DCs targeted with hD1-KLH induced stronger proliferative responses than DCs pulsed with KLH (Figure 6).

hD1-KLH targeted to DCs activates both major histocompatibility complex (MHC) class I– and II–restricted naive T cells recognizing KLH epitopes

To evaluate whether hD1-KLH, besides triggering recall responses, is able to activate naive T cells, hD1-KLH was tested in an autologous in vitro culture system. PBLs from a healthy donor were stimulated by repetitive coculturing with hD1-KLH–pulsed autologous DCs. IL-10 was added to the cultures to stimulate expansion of $CD8^+$ T cells.[25] After 5 rounds of stimulation, PBLs were cocultured with DCs pulsed with KLH or hD1 to evaluate KLH-specific proliferative responses. The results demonstrate that hD1-KLH–targeted DCs activated KLH-specific naive T cells (Figure 7). Presentation of KLH epitopes was mediated by MHC class I and class II, since both class I and class II blocking antibodies significantly reduced the proliferative response, while an antibody control did not. DCs pulsed with hD1 antibody did not induce proliferative responses by the PBLs (Figure 7), demonstrating that the T-cell response is specific for KLH epitopes rather than epitopes contained within the hD1 antibody.

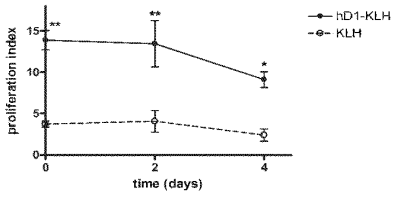


Figure 6. Antigen targeted to DC-SIGN is presented for at least 4 days. iDCs were incubated with medium, 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 37°C. Subsequently, iDCs were washed and cultured for 0, 2, or 4 days before addition of autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. Four days after addition of PBLs, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from KLH according to ANOVA and Bonferroni test: $^*P < .05$; $^{**}P < .01$.

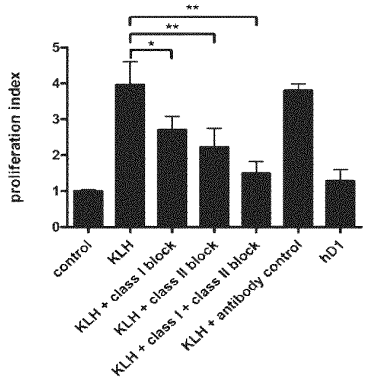


Figure 7. hD1-KLH–treated DCs activate naive T cells recognizing KLH epitopes. iDCs derived from a healthy donor were incubated with medium (control), 10 μg/mL KLH, or 10 μg/mL hD1 for one hour at 37°C. Subsequently, cells were washed and matured with LPS. mDCs were cocultured with autologous PBLs that had been repeatedly stimulated with DCs treated with hD1-KLH as described in "Patients, materials, and methods." Some cocultures of KLH-pulsed mDCs and PBLs were supplemented with w6/32 antibody (KLH + class I block), IVA-12 (KLH + class II block), both w6/32 and IVA-12 (KLH + class I + class II block), or total mouse IgG (KLH + antibody control). Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference according to ANOVA, followed by the Student-Newman-Keuls test: $^*P < .01$; $^{**}P < .001$.

Discussion

In the present study, we explored the capacity of the type II C-type lectin DC-SIGN to function as a target receptor for vaccination purposes. The results demonstrate that antibody-mediated targeting of antigen to human DC-SIGN was 100-fold more efficient than pulsing DCs with antigen, and that antigen epitopes were presented for at least 4 days after targeting.

A critical step in designing a suitable targeting construct is the choice of targeting antibody. Various antibodies directed against the same receptor might bind distinct receptor epitopes, thus influencing biologic outcome. For example, not all antibodies directed against DC-SIGN are internalized following binding,[13] and the anti-MR antibody PAM-1 induces maturation of iDCs, in contrast to a different isotype-matched anti-MR antibody.[26] The mouse antihuman DC-SIGN antibody AZN-D1 has been previously described to be internalized by DCs,[13] a prerequisite for successful targeting. However, the use of murine antibodies in humans presents numerous problems, including a short half-life and high immunogenicity.[27] We were able to graft the CDR of AZN-D1 hypervariable domains onto a human composite IgG2/IgG4 antibody without loss of binding characteristics. The resulting hD1 antibody exhibited a similar binding affinity for DC-SIGN as AZN-D1, and did not induce maturation of iDCs (data not shown). hD1 binding induced rapid internalization of DC-SIGN, while internalization did not result in a lasting down-regulation of DC-SIGN expression (data not shown), thus minimizing the effect a therapeutic intervention might have on the biologic function of DC-SIGN. hD1 rapidly targeted antigen to the DC lysosomal compartment resulting in antigen presentation, and did not induce hD1-specific proliferative responses in the naive T-cell stimulation experiments. Thus, humanization of AZN-D1 resulted in an effective targeting antibody, and will facilitate introduction into clinical trials.

Vaccination strategies aimed at inducing cytotoxic T-cell help, such as antitumor therapies, require antigen presentation in the

Samsung Bioepis Exhibit No. 1002
Page 7629

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context of MHC class I. In addition, the induction of CTL responses requires bystander $CD4^+$ T-cell help.[28] Targeting DCs with hD1-KLH resulted in the activation of naive T cells in the context of both MHC class I and II. Although the major route for presentation of exogenous antigens is via class II, presentation via class I can occur via the process of cross-presentation. This process provides internalized proteins access to cytosolic proteasomes and their derived peptides access to the endoplasmatic reticulum (ER)–based class I processing machinery.[29] Particulate antigens taken up by phagocytosis have access to this machinery since phagosomes fuse with the ER soon after or during their formation.[30] Particulate antigens are more efficiently cross-presented than soluble antigens.[31,32] However, a recent study by Ackerman et al reveals that internalized soluble proteins can escape proteolysis and also gain access to the lumen of the ER.[33] This might explain how antibody-mediated targeting of the type I C-type lectins MR[7] and DEC-205[10] results in class I responses. Although we cannot exclude the possibility that, besides receptor-mediated endocytosis, a portion of our chimeric protein was taken up via macropinocytosis, our current findings strongly suggest that antibody-mediated targeting of DC-SIGN results in cross-presentation.

The technique commonly used in current clinical trials to load DCs with antigens involves ex vivo incubation with MHC class I and II binding peptides. Other techniques involve loading with tumor lysates or apoptotic tumor cells, and the introduction of genetic material to drive expression of specific antigens by the DC itself.[2] Ideally, vaccines should deliver antigens to the DCs in vivo. Antibody-mediated targeting of antigens to DC surface receptors to stimulate antigen presentation in vivo has been shown to be far more potent than immunization with antigen in complete Freund adjuvant or splenic DCs pulsed with antigen ex vivo.[6] However, mere targeting of antigens to DCs is not sufficient for induction of immunity. Antibody-mediated targeting of antigen to DEC-205 in mice leads to tolerance, and coadministration of a DC maturation stimulus is required to induce immunity.[6,10,11] These findings are consistent with DC-based vaccination studies in humans, showing that DC maturation is a prerequisite for induction of immunity.[20] Vaccination strategies for transplantation, allergy, autoimmunity, and chronic inflammatory diseases could exploit the finding that targeted iDCs induce tolerance. However, strategies aimed at inducing immunity will require a combination of antigens and DC activation factors. Agents that have been shown to activate DCs in vivo include anti-CD40 antibody,[11] α-galactosylceramide,[34] and the Toll-like receptor ligands LPS[35] and cytosine-phosphate-guanosine (CpG) oligonucleotides.[36] A better understanding of differences in DC subsets, their activation pathways, and antigen uptake receptors will provide the information necessary for development of effective vaccines.

In the present study, we used a neoantigen to study targeting efficiency. A major challenge in cancer therapy is to break immunologic tolerance to tumor-associated self-antigens. Tolerance can be the result of clonal deletion, active suppression of antigen-specific T cells by regulatory T cells, or inadequate activation stimuli provided by tumor cells upon antigen presentation, resulting in T-cell anergy.[37] One way of breaking tolerance is by disruption of negative regulatory mechanisms directly at the T-cell level. Antibody-mediated blockade of the cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) breaks tolerance against moderately immunogenic tumors[38] and improves the efficiency of tumor cell–based vaccines against poorly immunogenic tumors.[39] Depletion of $CD25^+$ regulatory T cells prior to blocking of CTLA-4 improves vaccine efficiency even further.[40] A second way of breaking tolerance is to recruit DCs as potent APCs. DCs can overcome the heightened threshold of anergic T cells[41] and restore responsiveness of tolerogenic tumor-specific T cells[42] in vitro. Moreover, studies in mice demonstrate that DC-based vaccines can break tolerance against (tumor-associated) self-antigens,[43-47] resulting in regression of established tumors.[47] Thus, a combination of antibody-mediated strategies targeting tumor-associated antigens to DCs and strategies modulating regulatory mechanisms at the T-cell level might provide effective cancer therapies.

In conclusion, antibody-mediated targeting of antigens to DC surface receptors represents an exciting way to induce immune responses. Our results demonstrate efficient delivery of antigen to DCs via DC-SIGN, resulting in naive as well as recall responses by T cells. These data expose DC-SIGN as a promising target molecule for antibody-mediated antigen delivery to DCs.

Acknowledgments

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Page 7630

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Samsung Bioepis Exhibit No. 1002
Page 7631

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Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti–DC-SIGN antibody

Paul J. Tacken, I. Jolanda M. de Vries, Karlijn Gijzen, Ben Joosten, Dayang Wu, Russell P. Rother, Susan J. Faas, Cornelis J. A. Punt, Ruurd Torensma, Gosse J. Adema and Carl G. Figdor

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Samsung Bioepis Exhibit No. 1002
Page 7632

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The Efficacy and Safety of Rituximab in Patients With Active Rheumatoid Arthritis Despite Methotrexate Treatment

Results of a Phase IIb Randomized, Double-Blind, Placebo-Controlled, Dose-Ranging Trial

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Objective. **To examine the efficacy and safety of different rituximab doses plus methotrexate (MTX), with or without glucocorticoids, in patients with active rheumatoid arthritis (RA) resistant to disease-modifying antirheumatic drugs (DMARDs), including biologic agents.**

Methods. **A total of 465 patients were randomized into 9 treatment groups: 3 rituximab groups (placebo [n = 149], 500 mg [n = 124], or 1,000 mg [n = 192] on days 1 and 15) each also taking either placebo glucocorticoids, intravenous methylprednisolone premedication, or intravenous methylprednisolone premedication plus oral prednisone for 2 weeks. All patients received MTX (10–25 mg/week); no other DMARDs were permitted.**

Results. **Significantly more patients who received 2 500-mg or 2 1,000-mg infusions of rituximab met the American College of Rheumatology 20% improvement criteria (achieved an ACR20 response) at week 24 (55% and 54%, respectively) compared with placebo (28%; $P < 0.0001$). ACR50 responses were achieved by 33%, 34%, and 13% of patients, respectively ($P < 0.001$), and ACR70 responses were achieved by 13%, 20%, and 5% of patients ($P < 0.05$). Changes in the Disease Activity Score in 28 joints (−1.79, −2.05, −0.67; $P < 0.0001$) and moderate to good responses on the European League Against Rheumatism criteria ($P < 0.0001$) reflected the ACR criteria responses. Glucocorticoids did not contribute significantly to the primary efficacy end point, ACR20 response at 24 weeks. Intravenous glucocorticoid premedication reduced the frequency and intensity of first infusion–associated events; oral glucocorticoids conferred no additional safety benefit. Rituximab was well tolerated; the type and severity of infections was similar to those for placebo.**

Conclusion. **Both rituximab doses were effective and well tolerated when added to MTX therapy in patients with active RA. The primary end point (ACR20 response) was independent of glucocorticoids, although intravenous glucocorticoid premedication improved tolerability during the first rituximab infusion.**

Although cytokine-specific biologic agents have improved the treatment of rheumatoid arthritis (RA), some patients have an inadequate response to such therapies and, in clinical practice, most have only a

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Samsung Bioepis Exhibit No. 1002
Page 7633

partial response (1). The frequency of remission has remained <50% for patients with established RA, many of whom display "moderate" disease activity, according to their Disease Activity Score (DAS) (2). In order to achieve additional significant gains in RA therapy, new targets need to be identified. One such target is the B cell (3).

Rituximab is a monoclonal antibody that selectively depletes CD20+ B cells via 3 putative mechanisms: antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and promotion of CD20+ B cell apoptosis (4–6). CD20 is not expressed on stem cells or plasma cells (7,8); this is consistent with the observation that B cell recovery and Ig production were not compromised after a single course of rituximab (9).

Rituximab has shown efficacy in the treatment of CD20+ B cell non-Hodgkin's lymphoma (NHL) (10). More recently, a role for rituximab and B cell modulation has been investigated in rheumatoid factor (RF)–positive patients with active RA. In a phase IIa study, 2 1,000-mg infusions of rituximab (combined with methotrexate [MTX] and a 2-week treatment with glucocorticoids) significantly improved RA symptoms for up to 1 year compared with MTX alone (9). Moderate to good responses according to the European League Against Rheumatism (EULAR) criteria were also evident at 2 years in 33% of the rituximab plus MTX group (11).

To date, no studies have investigated the influence of the rituximab dosage or the role of glucocorticoids in the clinical efficacy and/or tolerability of rituximab regimens in RA. The objective of this study was therefore to examine these variables in patients with active RA who had an inadequate response to disease-modifying antirheumatic drugs (DMARDs) other than MTX or biologic agents, and who currently had an inadequate clinical response to MTX.

PATIENTS AND METHODS

Role of the funding source. This study was funded by Roche, and Roche scientists contributed to the study design, collection of data, and data analysis. The manuscript was written by Dr. Emery. The agreement to submit the manuscript and the approval of the content were made jointly by the authors.

Study design. This phase IIb, randomized, double-blind, double-dummy, placebo-controlled, international multifactorial trial of 9 different treatment regimens examined 2 dosage levels (and rituximab placebo) of both rituximab and glucocorticoids in a 3 × 3 configuration (Figure 1) with concomitant MTX in patients with active RA. Patients were evaluated at 4-week intervals between baseline and week 24.

Patients. Outpatients were eligible for study if they were between 18 and 80 years of age and had presented at least 6 months prior to randomization with moderate or severe RA

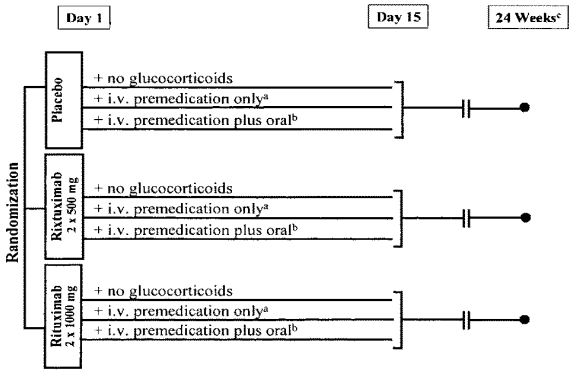


Figure 1. Study design. All groups received weekly methotrexate 10–25 mg parenterally or orally. a = 100 mg methylprednisolone administered intravenously (IV) prior to rituximab infusions on days 1 and 15. b = 100 mg methylprednisolone administered IV prior to rituximab infusions on days 1 and 15, and prednisone administered orally at 60 mg on days 2–7 and at 30 mg on days 8–14. c = primary end point: percentage of patients who met the American College of Rheumatology 20% improvement criteria at week 24.

(diagnosed according to the American College of Rheumatology [ACR; formerly, the American Rheumatism Association] revised criteria [22]) despite ongoing treatment with MTX at a dosage of 10–25 mg/week (orally or parenterally) for at least 12 weeks before randomization, with a stable dosage during the last 4 weeks. Active disease was defined as a swollen and tender joint count ≥8 and either an erythrocyte sedimentation rate ≥28 mm/hour or a C-reactive protein (CRP) serum level ≥1.5 mg/dl.

Patients must have failed prior treatment, manifesting as a lack or loss of response to treatment with at least 1 but not more than 5 DMARDs (other than MTX) and/or biologic response modifiers. Patients discontinued DMARDs (except MTX) and biologic therapy at least 4 weeks before randomization and discontinued infliximab, adalimumab, or leflunomide at least 8 weeks before randomization. Patients who were already taking oral glucocorticoids (≤10 mg/day of prednisone or equivalent) were included if the dosage had been stable for at least 4 weeks before trial entry, and patients who had received intraarticular or parenteral glucocorticoids were included, provided that the most recent administration was >4 weeks before screening. Concomitant treatment with nonsteroidal antiinflammatory drugs was permitted if the dosage had been stable for at least 2 weeks prior to entry. Concomitant treatment with any DMARD (other than MTX), anti–tumor necrosis factor α, or other biologic therapy was prohibited.

Patients were excluded if they had significant systemic involvement secondary to RA, evidence of significant other illnesses or laboratory abnormalities, a history of severe allergic or anaphylactic reactions to humanized or murine monoclonal antibodies, or previous treatment with rituximab or any lymphocyte-depleting therapies. In addition, patients were excluded if they had a history of recurrent significant infection.

The study was performed in accordance with the Declaration of Helsinki. All participating sites received ap-

Samsung Bioepis Exhibit No. 1002
Page 7634

Table 1. Randomization of trial medication and disposition of patients at 24 weeks*

	Treatment†		
	Rituximab placebo, RF+/RF−	Rituximab 2 500-mg doses, RF+/RF−	Rituximab 2 1,000-mg doses, RF+/RF−
Glucocorticoid placebo	42/21	41/0	43/22
IV glucocorticoid premedication‡	42/0	41/0	42/20
IV glucocorticoid premedication + oral glucocorticoid§	44/0	42/0	43/22
Total no. of patients randomized¶	149	124	192
Patients withdrawn, no. (%)	52 (35)	11 (9)	27 (14)
Insufficient response	46 (31)	8 (6)	16 (8)
Adverse events	0	3 (2)	6 (3)
All other reasons#	6 (4)	0	5 (3)
Completed 24 weeks, no. (%)	97 (65)	113 (91)	165 (86)

* RF = rheumatoid factor; IV = intravenous.
† Rituximab or rituximab placebo was administered as 2 infusions: 1 on day 1 and 1 on day 15. All patients received 10–25 mg of methotrexate orally or parenterally on a weekly basis.
‡ Methylprednisolone 100 mg IV on days 1 and 15.
§ Methylprednisolone 100 mg IV on days 1 and 15 plus oral prednisone 60 mg on days 2–7 and 30 mg on days 8–14 (total oral prednisone dose 570 mg).
¶ Patients received at least 1 dose of rituximab.
In the rituximab placebo and rituximab groups receiving 2 1,000-mg infusions, 4 patients (3%) and 4 patients (2%), respectively, refused treatment.

proval from their governing institutional review board (or equivalent). See Appendix A for additional members of the Dose-Ranging Assessment: International Clinical Evaluation of Rituximab in Rheumatoid Arthritis (DANCER) Study Group and their locations. All patients provided written informed consent. The primary study population for efficacy analysis was composed of RF-positive patients. A limited number of RF-negative patients were also included for an initial exploratory analysis of the safety of rituximab in this group.

Study treatment. Rituximab was administered by intravenous (IV) infusion in RF-positive patients: placebo, 500 mg or 1,000 mg on days 1 and 15 (total dose 0 mg, 1,000 mg, and 2,000 mg). Glucocorticoids were administered as placebo methylprednisolone, given IV 30–60 minutes before the infusion of rituximab (or rituximab placebo) on days 1 and 15, premedication methylprednisolone 100 mg, given IV on days 1 and 15 (250 mg prednisone equivalent), or premedication methylprednisolone 100 mg, given IV on days 1 and 15 plus 60 mg of oral prednisone on days 2–7 and 30 mg on days 8–14 (total glucocorticoid dose 820 mg prednisone equivalent) (Table 1). RF-negative patients received either placebo or rituximab (2 1,000-mg infusions), with or without glucocorticoids. All patients received a weekly regimen of MTX (10–25 mg orally or parenterally) with folate (≥5 mg/week).

Trial end points. The primary end point of the trial was the proportion of RF-positive patients who met the ACR 20% improvement criteria (achieved an ACR20 response) (defined as a 20% improvement in the tender and swollen joint counts and a 20% improvement in 3 of the 5 other ACR improvement criteria) (13) at week 24. Secondary and exploratory analyses examined group differences in ACR50 and ACR70 responses, DAS 28-joint assessment for swelling and tenderness (DAS28) (14), EULAR responses (15), fatigue, as measured by the Functional Assessment of Chronic Illness Therapy–Fatigue (FACIT-F) subscale (16), and the effect on individual parameters of the ACR improvement criteria. The level of physical functioning and disability was assessed using the disability index (DI) of the Health Assessment Questionnaire (HAQ) (17).

Safety outcomes were expressed as the incidence of adverse events, graded according to National Cancer Institute (NCI) Common Toxicity Criteria (CTC), version 2.0 (18). CD19+ counts, measured by fluorescence-activated cell sorting, were used as a marker of the onset and duration of peripheral B cell depletion (7). Effects on Ig levels, protective antibody titers, and human antichimeric antibody (HACA) levels were also evaluated.

Statistical analysis. The trial was planned to recruit 440 patients: 40 RF-positive patients per group (a total of 360 patients), who constituted the primary efficacy population, and 20 RF-negative patients in 4 of the 9 groups (a total of 80 patients), for exploratory analysis. Using a 2-sided 5% significance level, it was calculated that a sample size of 360 RF-positive patients would provide at least 80% power to detect differences between the proportions of patients who achieved an ACR20 response in the rituximab 2 1,000-mg and 2 500-mg infusion groups, as well as dose-response trends across the 3 pooled rituximab groups. Randomization was stratified by region (US versus non-US) and by RF status. Steroids were included in the main effects model of ACR20 response at 24 weeks to determine whether a significant effect of the steroid regimen had been achieved. The study was powered to test for an association between ACR20 response at week 24 and rituximab treatment while controlling for corticosteroid usage and region (US or non-US) for RF-positive patients, using a logistic regression model (main effects model). It was not powered to determine any individual effects of these controlling factors.

Primary analyses were based on intent-to-treat (ITT), with nonresponders imputed for all categorical end points (e.g., ACR and EULAR responses), and the last observation was carried forward for continuous variables (e.g., ACR improvement criteria and DAS28). All patients who dropped out of the study were considered nonresponders for purposes of categorical end points. A main-effect logistic regression model

Samsung Bioepis Exhibit No. 1002
Page 7635

Table 2. Patient demographics and baseline disease characteristics*

	Rituximab placebo	Rituximab 2 500-mg infusions	Rituximab 2 1,000-mg infusions
Safety population, no.	149	124	192
Demographics			
Female, %	80	83	80
Age, mean years	51.1	51.4	51.1
Duration of RA, mean years	9.3	11.1	10.8
Previous DMARDs (other than MTX), mean no.	2.2	2.5	2.5
Prior anti-TNFα treatment, %	26	33	28
MTX dose, mean mg/week	15.6	16.0	14.9
US patients, %	33	33	34
Primary ITT population, no.	122	123	122
Disease characteristics			
Swollen joint count, mean no.	21	22	22
Tender joint count, mean no.	35	33	32
Baseline HAQ DI score, mean	1.7	1.8	1.7
RF, mean IU/liter	437	421	437
ESR, mean mm/hour	40	45	41
Serum CRP, mean mg/dl	3.3	3.2	3.0
DAS28, mean	6.8	6.8	6.7

* RA = rheumatoid arthritis; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; TNFα = tumor necrosis factor α; ITT = intent to treat; HAQ = Health Assessment Questionnaire; DI = Disability Index; RF = rheumatoid factor; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints.

was used to evaluate ACR20 response at week 24 in the primary efficacy population across all study groups, with terms for rituximab, glucocorticoid usage, and region (US or non-US). This model also studied any effect of adding RF-negative patients to the analysis population. Contrast tests were conducted for rituximab 2 1,000-mg infusions versus placebo, and rituximab 2 500-mg infusions versus rituximab 2 1,000-mg infusions, in addition to a dose-response relationship in the ACR20 response across the upper- and lower-dose rituximab and placebo groups. No comparisons between the rituximab 2 500-mg infusions and placebo were originally planned.

RESULTS

Patient demographics and characteristics. A total of 465 patients were treated, 380 RF-positive patients and 85 RF-negative patients; this constituted the safety population (Table 1). Patients in each of the rituximab groups were generally well matched in terms of demographic parameters and region, baseline disease characteristics, and previous DMARD/biologic treatments (Table 2). Antimalarial agents had been taken by 38–68% of patients across treatment groups, sulfasalazine by 38–68% and immunosuppressive agents (cyclosporine, azathioprine, and/or leflunomide) by 24–44%. Overall, 29% of patients had previously taken biologic agents (infliximab, adalimumab, etanercept, or anakinra), with no significant differences between the randomized groups.

Of the 465 patients, 65% in the placebo group, 91% in the rituximab 2 500-mg infusions group, and 86% in the rituximab 2 1,000-mg infusions group completed the 24-week primary analysis period (Table 1). Fewer patients discontinued rituximab than placebo as a result of insufficient therapeutic response, but rituximab was associated with more withdrawals because of adverse events. Nine patients (all in rituximab groups) withdrew for safety reasons (mainly as a result of infusion-related events). One patient in the 2 500-mg rituximab infusions plus IV glucocorticoid group died due to a cerebrovascular accident.

The primary ITT efficacy population was composed of 367 RF-positive patients; the remaining RF-positive patients were excluded from this analysis for reasons related to accidental unblinding, drug dispensing errors, and unverifiable data.

Efficacy outcomes. The proportion of patients in the primary ITT efficacy population who achieved an ACR20 response at 24 weeks, the primary end point of the trial, was significantly greater in each of the rituximab groups than in the placebo group ($P < 0.0001$) (Figure 2A). At week 24, 55% of the patients in the rituximab 2 500-mg infusions group and 54% of those in the 2 1,000-mg infusions group had achieved an ACR20 response, compared with 28% in the placebo group. There was no statistically significant difference in the odds of achieving an ACR20 response between the groups taking 2 1,000-mg infusions of rituximab versus 2 500-mg infusions of rituximab (odds ratio 0.93, $P = 0.768$). Analyses of the proportions of patients who achieved an ACR50 or ACR70 response showed a similar pattern. Compared with placebo, a significantly greater proportion of patients treated with either dosage

Samsung Bioepis Exhibit No. 1002
Page 7636

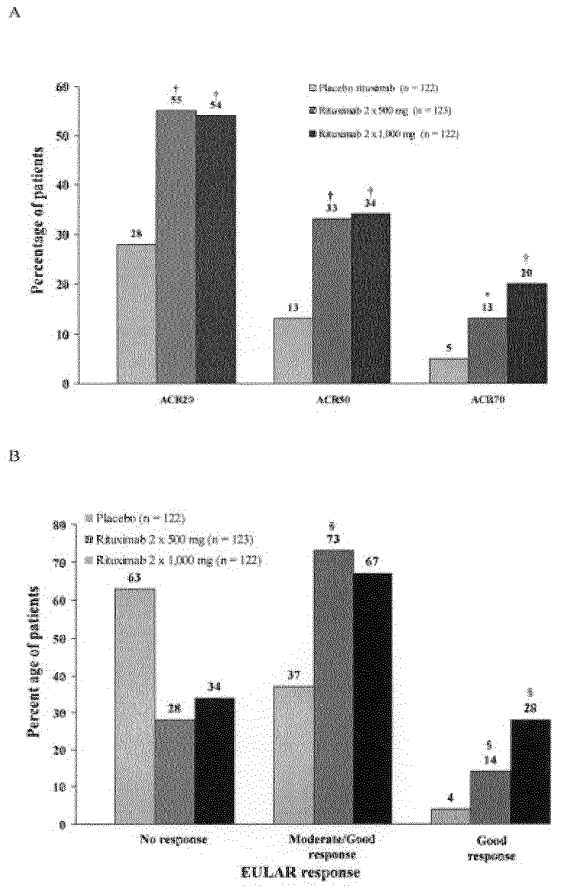


Figure 2. Patients who experienced responses according to **A,** the American College of Rheumatology 20% (ACR20), 50% (ACR50), and 70% (ACR70) improvement criteria and **B,** the European League Against Rheumatism (EULAR) criteria at 24 weeks. Results are expressed using pooled data for glucocorticoid dose. Patients who withdrew or who had insufficient data from which to calculate an ACR score were classified as nonresponders. * = $P = 0.029$; † = $P \leq 0.001$; § = $P < 0.0001$, versus placebo.

of rituximab achieved an ACR50 response ($P \leq 0.001$ versus placebo in each rituximab dosage group) and an ACR70 response ($P = 0.029$ for rituximab 2 500-mg infusions group and $P \leq 0.001$ for rituximab 2 1,000-mg infusions group). Although ACR20 and ACR50 responses were similar for both rituximab dosages, ACR70 responses were numerically more frequent in the rituximab 2 1,000-mg infusions group than in the 2 500-mg infusions group (Figure 2A).

Primary analysis within the main-effect model showed that glucocorticoid treatment had no significant effect on ACR20 response ($P = 0.166$). Dose-contrast tests between individual glucocorticoid treatments showed that the probability of achieving an ACR20 response in any of the main treatment groups was not significantly different for glucocorticoid placebo versus IV glucocorticoids ($P = 0.62$) or IV and oral glucocorticoids ($P = 0.18$). Patients who received glucocorticoids were, however, more likely to achieve an ACR20 response by week 4 than those who received no glucocorticoids. Consequently, since glucocorticoid treatment was not a prerequisite for the efficacy of rituximab, efficacy results are expressed using pooled glucocorticoid dose cohorts within each rituximab group.

The time course for achieving an ACR response showed early separation of rituximab from placebo. The proportion of patients who achieved an ACR20 response in the placebo group leveled off at ~30% at week 4, while in the rituximab groups, it continued to increase, leveling off at ~55% at 12 weeks. The same was essentially true for ACR50 and ACR70 responses, except in the rituximab 2 1,000-mg infusions group, in which ACR70 was seen in 10% of patients at week 12 and increased to 20% at week 24, suggesting a time-related effect on achievement of higher-level responses at this dose.

Changes in the DAS28 at week 24 reflected ACR response findings. Analysis of variance showed the adjusted mean change in the DAS28 from baseline to be significantly greater in patients treated with rituximab 2 500-mg and 2 1,000-mg infusions (−1.79 and −2.05, respectively) than in patients treated with placebo (−0.67; $P < 0.0001$). Similarly, EULAR responses in patients treated with rituximab at either dosage were significantly greater than those seen in patients who received placebo (Figure 2B). Compared with placebo, moderate or good EULAR responses were achieved in a significantly greater proportion of patients treated with rituximab 2 500-mg infusions ($P < 0.0001$) and rituximab 2 1,000-mg infusions ($P < 0.0001$), with a trend toward a higher proportion of patients achieving a good EULAR response with 2 1,000-mg infusions of rituximab.

CRP levels fell by a mean of 1.7 mg/dl between baseline and week 24 in patients treated with the active rituximab regimens. The corresponding mean change in CRP in the rituximab placebo group was 0.1 mg/dl. These differences in favor of the active rituximab groups were observed after week 4 and continued to week 24. In patients who were RF-positive at baseline, the proportion with RF titers below the level of detection by week 24 was 1.6% in the placebo group and 12% and 9% in the rituximab 2 500-mg infusions and 2 1,000-mg infu-

Samsung Bioepis Exhibit No. 1002
Page 7637

Table 3. Most frequently reported adverse events in the safety population (n = 465)*

Adverse events	Rituximab placebo (n = 149)	Rituximab 2 500-mg infusions (n = 124)	Rituximab 2 1,000-mg infusions (n = 192)
All events	105 (70)	100 (81)	164 (85)
Exacerbation of RA	44 (30)	21 (17)	27 (14)
Headache	19 (13)	14 (11)	21 (11)
Nausea	13 (9)	8 (6)	19 (10)
URI	9 (6)	10 (8)	12 (6)
Nasopharyngitis	8 (5)	7 (6)	10 (5)
Arthralgia	5 (3)	5 (4)	11 (6)
Diarrhea	8 (5)	7 (6)	6 (3)
Fatigue	8 (5)	5 (4)	8 (4)
Hypertension	4 (3)	5 (4)	12 (6)
Rigors	3 (2)	5 (4)	13 (7)
Dizziness	6 (4)	4 (3)	10 (5)
Serious noninfection adverse events	2 (1)†	9 (7)‡	9 (5)§
Serious infections	2 (1)	0	4 (2)

* All adverse events affecting ≥5% of patients in any one group are shown. Values are the number (%). RA = rheumatoid arthritis; URI = upper respiratory tract infection.
† Food allergy and exacerbation of RA in 1 patient each.
‡ Cerebral infarction, fatal cerebrovascular accident unrelated to study treatment, convulsion, myocardial infarction, supraventricular tachycardia, drug hypersensitivity (occurring within 24 hours of rituximab infusion), failure of implant, lower limb fracture, and interstitial lung disease in 1 patient each.
§ Epilepsy, serotonin syndrome, chest pain, generalized edema (occurring within 24 hours of rituximab infusion), exacerbation of RA, status asthmaticus, intestinal obstruction, metrorrhagia, and thromboangiitis obliterans in 1 patient each.

sions groups, respectively. This reflected a mean increase in total RF titers in the placebo groups (ranging from 7.1% to 37.4% among placebo groups) compared with a mean decrease in the active rituximab groups (ranging from 11.5% to 47.9% among rituximab groups).

Patient-reported outcomes mirrored the improvements in clinical and laboratory findings in the primary ITT population. Changes in the mean DI scores of the HAQ between baseline and week 24 were −0.43 and −0.49 in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively, compared with −0.16 in the rituximab placebo group. The proportions of patients with an improvement of −0.22 from baseline (minimum clinically important difference [MCID] [19]) were 63%, 67%, and 34%, respectively in these 3 groups. Furthermore, changes in fatigue, measured as the percentage improvement in the FACIT-F score between baseline and week 24, were 20% and 28% in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively, compared with 4% in the placebo group. In both rituximab groups, the mean change from baseline in the FACIT-F score exceeded 4 points, which is the MCID (16). This was not the case in the placebo group.

The inclusion of data from RF-negative patients in the analyses did not alter the primary outcome. In the combined RF-positive and RF-negative population, 52% of the rituximab 2 1,000-mg doses group achieved an ACR20 response at week 24 compared with 32% of the placebo group. An exploratory analysis of RF-negative patients alone proved inconclusive, since it was confounded by an unusually high placebo response (52% versus 48% in the rituximab group) and a relatively small sample size (21 and 63 patients, respectively).

There was a significant difference at week 24 between the absolute proportions of US and non-US patients who achieved an ACR20 response across treatment groups, including placebo ($P = 0.002$), with an ACR20 response being more frequent in the non-US region. Between regions, the efficacy of rituximab relative to placebo (i.e., the difference between placebo and active drug responses) was similar, and consequently, there was no significant interaction between rituximab and region ($P = 0.797$). An exploratory analysis of the 2 regions showed that the only major baseline difference between the US and non-US populations was the proportion of patients previously exposed to biologic therapy (55% of US versus 23% of non-US patients). While there was no identified interaction between prior use of biologic therapies and rituximab treatment, ACR20 response rates were generally lower in patients with prior exposure to biologic therapies.

Safety outcomes. Overall, 70% of patients treated with placebo reported at least 1 adverse event at any time during the study period, compared with 81% and 85% of patients in the rituximab 2 500-mg and 2

Samsung Bioepis Exhibit No. 1002
Page 7638

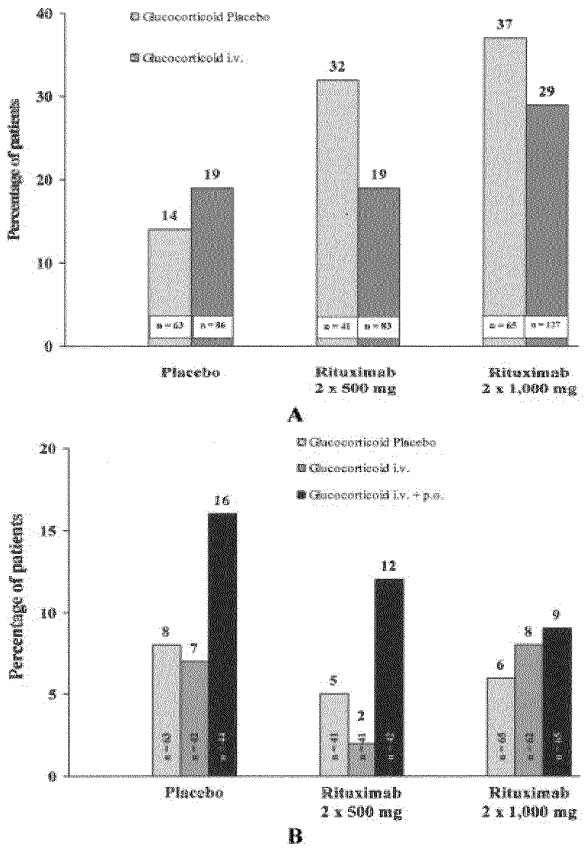


Figure 3. Effect of glucocorticoids on the frequency of acute infusion reactions after **A,** the first infusion and **B,** the second infusion. IV = intravenous; PO = by mouth.

1,000-mg infusions groups, respectively. The majority (82%) of events in each group were mild to moderate in severity (NCI CTC grades 1–2). A similar proportion of patients reported severe (NCI CTC grade 3) events in each group (18% of patients receiving placebo and 17% and 18% of patients in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively). The most frequently reported adverse events in each treatment group are shown in Table 3 and were mainly associated with the first infusion. Exacerbation of RA, reported as an adverse event, distinguished rituximab placebo from both doses of active rituximab, reflecting the efficacy of rituximab relative to placebo. There were no unexpected adverse events in the RF-negative subgroup.

Infusion-associated events. Infusion-associated events (defined as any adverse event that occurred during or within 24 hours of rituximab infusion) occurred with the first infusion in 18% of patients who received placebo and in 31% and 38% of patients who received rituximab 2 500-mg and 2 1,000-mg infusions, respectively.

Acute infusion reactions. Symptoms or signs suggesting an acute infusion reaction (pruritus, fever, urticaria/rash, chills, pyrexia, rigors, sneezing, angioneurotic edema, throat irritation, cough and bronchospasm, hypotension, or hypertension) were more commonly associated with the first infusion of rituximab compared with placebo. Overall, acute infusion reactions were reported at the first infusion in 17% of placebo-treated patients compared with 23% and 32% of patients who received 2 500-mg or 2 1,000-mg rituximab, respectively. However, premedication with IV glucocorticoid significantly reduced both the incidence and severity of acute infusion reactions. Without glucocorticoid premedication, these events were reported by 14% of the placebo group, 32% of the rituximab 2 500-mg infusions group, and 37% of the rituximab 2 1,000-mg infusions group, as compared with 19%, 19%, and 29% of these groups, respectively, following IV glucocorticoid premedication (Figure 3A).

The number of patients who experienced these symptoms following the second infusion of the treatment course was substantially lower in all groups, irrespective of glucocorticoid premedication (Figure 3B). The addition of 2 weeks of oral prednisone treatment did not appear to provide any further reduction of acute infusion reactions with the second infusion.

Two adverse events occurred within the first 24 hours of the first infusion: 1 case of drug hypersensitivity reaction occurred in the rituximab 2 500-mg infusions group, and 1 case of generalized edema occurred in the rituximab 2 1,000-mg infusions group. Both events occurred in patients who had not received IV glucocorticoid premedication. Five patients (2.6%) in the 2 1,000-mg infusions rituximab group were withdrawn from treatment because of acute infusion-associated events following the first infusion. Four of these patients had not received glucocorticoid premedication.

Infection-related adverse events. Adverse events classed as infections and infestations were reported in 28% of the placebo group and in 35% of each of the active rituximab groups. The type and severity of infections were similar across the placebo and active rituximab groups (the most common being respiratory tract infections, urinary tract infections, and nasopharyngitis),

Samsung Bioepis Exhibit No. 1002
Page 7639

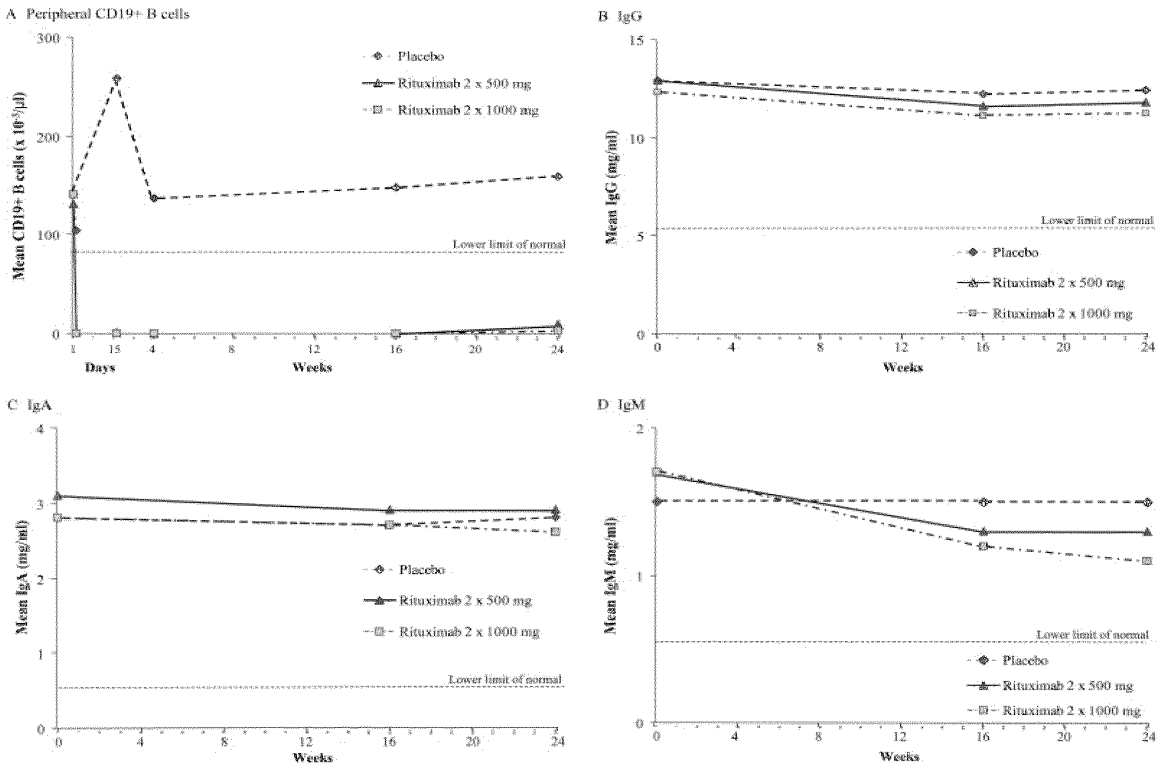


Figure 4. Levels of **A,** peripheral blood CD19+ B cells, **B,** IgG, **C,** IgA, and **D,** IgM during the 24-week treatment period.

so the pattern of infections did not distinguish between placebo- and rituximab-treated patients.

Six serious infections were reported. Two (1%)occurred in the placebo group (pneumonia and respiratory tract infection) and 4 (2%) in the rituximab 2 1,000-mg infusions group (2 cases of pyelonephritis, and 1 each of bronchitis and epiglottitis). These events resolved without sequelae. No serious infections occurred in the rituximab 2 500-mg infusions group. The rate of serious infections per 100 patient-years was 3.19 for the rituximab placebo group, 0 for the 2 500-mg infusions of rituximab group, and 4.74 for the 2 1,000-mg infusions of rituximab group.

Serious adverse events. A total of 26 serious adverse events were reported, 4 (3%) in the placebo group (3%), 9 in the rituximab 2 500-mg infusions group (7%), and 13 in the rituximab 2 1,000-mg infusions group (7%), with no consistent pattern or signal. Of these, 20 were noninfection events in 2, 9, and 9 patients in the placebo, rituximab 2 500-mg, and rituximab 2 1,000-mg infusions groups (1%, 7%, and 5%, respectively) (Table 3). Five events were nervous system disorders, while others were distributed evenly across body systems in no discernible pattern. There was 1 fatal event, which occurred in the rituximab 2 500-mg infusions group. A 73-year-old woman died of a cerebral infarction 167 days after the first infusion of rituximab. This patient had a history of atrial fibrillation, hypertension, diabetes mellitus, and hyperlipidemia; at the time of enrollment into the trial, she was receiving concomitant celecoxib, MTX, folic acid, lisinopril, atorvastatin, and sertraline.

Pharmacodynamics. In the placebo group, peripheral B cell levels remained stable around baseline levels throughout the study period, after an initial transient increase during the 2-week glucocorticoid dosing period between rituximab placebo infusions. Treatment with rituximab was associated with nearly complete

Samsung Bioepis Exhibit No. 1002
Page 7640

depletion of peripheral blood B cells, which persisted throughout the 24-week study period. Peripheral blood B cell recovery began after week 16 (Figure 4A).

Although peripheral blood B cell levels were depleted in rituximab-treated patients, mean and median Ig levels (IgG, IgA, and IgM) remained above the laboratory lower limit of normal at all times; the greatest change from baseline occurred in IgM (Figures 4B–D). Anti-tetanus titers remained stable.

Antibodies to the test agent, i.e., HACA (analyzed up to and including week 24), were detectable in 0.7%, 4.2%, and 2.7% of the placebo, rituximab 2 500-mg, and rituximab 2 1,000-mg infusions groups, respectively. There were no serious adverse events in the group of patients with HACAs. Moreover, 6 of the 10 rituximab-treated patients with positive HACAs at week 24 achieved ACR20 responses at that time.

DISCUSSION

Results of the DANCER trial have confirmed that a single course of rituximab, given as 2 infusions 2 weeks apart, is highly effective over 24 weeks in the treatment of active RA in patients who have shown an incomplete response to MTX. Followup of these patients beyond the primary end point is ongoing, and radiologic outcomes (not measured in this study) will be addressed in future studies. Rituximab has a novel mode of action that results in the depletion of B cells, and it is therefore distinct from other biologic therapies for RA that target T cells and their related cytokines. The present study corroborates earlier studies that support a role for B cells in the inflammatory processes that underlie RA (9,10,20–24).

Both rituximab dosages were effective, with 54–55% of patients achieving an ACR20 response by week 24, and a high proportion achieving ACR50 or ACR70 responses. Importantly, clinical improvements in rituximab-treated patients were reflected in patient- and physician-reported outcome measures, as well as in changes in laboratory parameters. Moreover, the beneficial effects of rituximab were often evident early in the course of treatment.

In general, the efficacy results from the DANCER study broadly mirror those from a phase IIa study of rituximab in 161 RF-positive RA patients (9), although in the latter, a higher ACR20 response rate was observed. A possible explanation for this discrepancy is the prior use of biologic therapies at baseline (an indication of refractory disease). In the phase IIa study, only ~10% of patients had been exposed to a biologic agent, whereas in the DANCER trial, the proportion was 30–40% in the active rituximab groups. A subanalysis of DANCER trial patients who received active rituximab with no prior exposure to biologic therapies determined an ACR20 response rate of 62% for this group, which is closer to previously reported rates (9). Previous use of biologic agents may also be a contributory factor to the lower absolute ACR response observed in US patients compared with non-US patients in this study. A greater proportion of US patients had received such treatment and may therefore have constituted a more refractory population. Nevertheless, rituximab was similarly effective relative to placebo both in patients who had received prior biologics and those naive to these therapies.

No dose-response relationship was established, based on the ACR20 criteria, for the 2 rituximab doses studied. There were, however, trends to indicate that the dose may influence the achievement of high-level response (i.e., ACR70 response and good EULAR response), and further evaluation of both rituximab doses is warranted.

The use of concomitant glucocorticoids with rituximab was based on the successful application of similar regimens in patients with NHL (10) and the initial trials in patients with RA (9,20,21). This trial has, however, established that glucocorticoids play no significant role in determining the primary end point of an ACR20 response at 24 weeks in RA patients treated with rituximab, although they may enhance the early response (up to 4 weeks) by virtue of their antiinflammatory properties. Consequently, the use of glucocorticoids does not appear to be a prerequisite for a clinical response to rituximab.

Both dosages of rituximab were generally well tolerated, with a low incidence of serious adverse events, including serious infections. The majority of adverse events were infusion associated, mild to moderate in intensity, and easily managed. Premedication with IV glucocorticoids did, however, reduce the incidence and severity of infusion-associated events following the first infusion of rituximab. The incidence of reactions following the second infusion was low in all groups, with 2 weeks of oral glucocorticoid treatment providing no additional safety benefit over IV glucocorticoid premedication alone. Overall, the incidence of infusion-related adverse events in RA patients appears to be lower than that observed in NHL patients (who may be prone to such events because of elevated levels of white blood cells or tumor lysis) (10,25).

All patients had depleted B cell levels following rituximab treatment, with peripheral blood B cell levels falling to below the lower limit of normal. Mean and

Samsung Bioepis Exhibit No. 1002
Page 7641

median Ig levels stayed within the normal range, but there was a downward trend specifically with IgM; however, there was no significant change in acquired immunity. Overall, the incidence of immunogenicity (HACAs) was low and had no apparent clinical relevance over the primary 24-week course of this trial, but patients continue to be monitored for this, together with all other pharmacodynamic parameters, including CD19 and Ig levels.

The DANCER study is a novel design for the study of RA: 9 treatment groups with a logistic regression main-effects model as its primary means of analysis. The complexity of the study was driven by a need to understand the effects of different components of a potential therapeutic regimen (i.e., rituximab dose and effects of glucocorticoids). Both objectives were met, but several factors must be taken into account when interpreting the results. First, extrapolation of these data to the larger RA population must be done with care, given the relatively small number of patients involved. In particular, specific comparison between any of the individual 9 groups is inappropriate; however, valuable guidance concerning both the use of glucocorticoids and the dosage of rituximab has been obtained. Second, it should be acknowledged that the present data represent just 24 weeks of followup; longer-term followup will be required to better characterize safety and, possibly, to identify potential differences between rituximab dosages. Last, with regard to the subset of RF-negative patients, small numbers of patients and a large placebo effect prevent the drawing of firm conclusions about the safety and efficacy of rituximab in this population.

In conclusion, both dosages of rituximab were highly effective and generally well tolerated when given with continuing MTX to patients with active RA. The study has also shown that concomitant glucocorticoids are not a prerequisite for the efficacy of rituximab in RA. However, premedication with IV glucocorticoids does appear to ameliorate acute infusion reactions. Both dosages of rituximab explored in this study warrant further differential exploration and longer-term followup.

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Page 7642

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APPENDIX A: MEMBERS OF THE DANCER STUDY GROUP

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Samsung Bioepis Exhibit No. 1002
Page 7643

D46

NATURAL HISTORY OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Abstract *Background.* Paroxysmal nocturnal hemoglobinuria (PNH), which is characterized by intravascular hemolysis and venous thrombosis, is an acquired clonal disorder associated with a somatic mutation in a totipotent hematopoietic stem cell. An understanding of the natural history of PNH is essential to improve therapy.

Methods. We have followed a group of 80 consecutive patients with PNH who were referred to Hammersmith Hospital, London, between 1940 and 1970. They were treated with supportive measures, such as oral anticoagulant therapy after established thromboses, and transfusions.

Results. The median age of the patients at the time of diagnosis was 42 years (range, 16 to 75), and the median survival after diagnosis was 10 years, with 22 patients (28 percent) surviving for 25 years. Sixty patients have died; 28 of the 48 patients for whom the cause of death is known died from either venous thrombosis or hemorrhage. Thirty-one patients (39 percent) had one or more episodes of venous thrombosis during their illness. Of the 35 patients who survived for 10 years or more, 12 had a spontaneous clinical recovery. No PNH-affected cells were found among the erythrocytes or neutrophils of the patients in prolonged remission, but a few PNH-affected lymphocytes were detectable in three of the four patients tested. Leukemia did not develop in any of the patients.

Conclusions. PNH is a chronic disorder that curtails life. A spontaneous long-term remission can occur, which must be taken into account when considering potentially dangerous treatments, such as bone marrow transplantation. Platelet transfusions should be given, as appropriate, and long-term anticoagulation therapy should be considered for all patients. (N Engl J Med 1995;333:1253-8.)

PAROXYSMAL nocturnal hemoglobinuria (PNH) is an acquired disorder of hematopoiesis characterized by intravascular hemolysis and manifested by episodes of hemoglobinuria and life-threatening venous thromboses.[1] The cellular abnormality in this disorder is caused by a somatic mutation in a totipotent hematopoietic stem cell.[2,3] The cells derived from the abnormal clone are deficient in all surface proteins normally attached to the cell membrane by a glycosylphosphatidylinositol anchor.[4,5]

PNH was first described as a distinct clinical entity in 1882.[6] The cardinal diagnostic test, introduced in the late 1930s, is Ham's test,[7] which is based on the increased sensitivity of PNH-affected erythrocytes to lysis by complement. Deficiency of an antigen known as the membrane inhibitor of reactive lysis (CD59) is largely responsible for the hemolysis[8,9] and is implicated in the tendency for patients to have thromboses.[9] In the past two years the biochemical defect underlying PNH has been pinpointed at an early step in the biosynthesis of glycosylphosphatidylinositol molecules — namely, the transfer of *N*-acetylglucosamine to phosphatidylinositol.[10-12] The protein required for this step is encoded by a gene, *PIG-A*, that is somatically mutated in patients with PNH.[13-17]

Despite the remarkable progress in our understanding of this disorder, treatment has remained largely supportive. The only potentially curative therapy currently available is bone marrow transplantation,[18,19] which is associated with substantial morbidity and mortality.[20] To determine whether the management of PNH can be improved, it is important to know its natural history. We report the results of a long-term study (up to 48 years after the diagnosis) of a group of 80 patients with PNH seen at one institution between 1940 and 1970. This study has uncovered some surprising information on the natural history of the disorder and has identified relatively simple therapies that may reduce the associated morbidity and mortality.

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METHODS

Patients

Eighty patients with PNH were referred to Hammersmith Hospital in London between 1940 and 1970. In all patients the diagnosis was established or confirmed by a positive Ham's test.[21] This group of patients was last described in 1972.[22] Follow-up data have been obtained by contacting the patients' primary physicians and reviewing death certificates.

Complications and Causes of Death

The complications and causes of death reported were either unequivocally diagnosable on clinical grounds or were diagnosed after death. If a complication was not confirmed, it is not reported here. Thus, the reported incidence of complications is likely to be an underestimate, because many of the patients were seen at Hammersmith Hospital only infrequently. We compared the survival of the patients with the survival of a control group matched for sex and age.

Analysis of Blood Samples

In 1993 venous blood samples were obtained from five patients in either acid–citrate–dextrose or EDTA for the performance of Ham's tests and flow cytometric studies of the expression of glycosylphosphatidylinositol-linked proteins on erythrocytes and white cells, as previously described.[23,24] By definition, normal cells express normal levels of glycosylphosphatidylinositol-linked proteins, whereas PNH-affected cells are deficient in all these proteins.

RESULTS

Follow-up Studies

Sixty of the 80 patients were known to have died, and 6 patients were lost to follow-up before 1972. The remaining 14 patients were alive when last seen between

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1975 and 1994, and 9 of the 14 were known to be alive in 1994.

Clinical Presentation

The presenting features or initial diagnoses were reported previously.[22] The median age at the time of diagnosis was 42 years (range, 16 to 75). Forty-three (84 percent) of the 51 patients for whom reliable information was available had episodes of hemoglobinuria as a chief symptom at some time during their illness.

Course of Illness and Survival

The course of the disease in each of the 80 patients is shown in Figure 1. From the actuarial survival curve for the group, we calculated a median survival of approximately 10 years (Fig. 2). Twenty-five years after the diagnosis, 58 patients (72 percent) had died, and 22 (28 percent) were alive. The median age at the time of death was 56 years (range, 20 to 84), with a median interval of 10 years (range, 0 to 48) between diagnosis and death. Patient 10 received the diagnosis of PNH in 1939, had a spontaneous remission in 1949, and died of bronchial carcinoma in 1987, 48 years after the diagnosis.

Cause of Death

For 12 of the 60 patients who died (Table 1), the cause of death was unknown. In 28 of the other 48 patients (58 percent), the cause of death (either thrombosis or hemorrhage attributable to thrombocytopenia) was directly related to PNH. In the other 20 patients (42 percent), the cause of death was unrelated to PNH.

Venous Thrombosis

Thirty-one patients (39 percent) were known to have had one or more episodes of venous thrombosis during the course of their illness (Table 2), and several patients had repeated thromboses. In many patients the thrombosis was either fatal or life-threatening, and it was often unheralded.

Spontaneous Remission

Twelve patients (15 percent) had spontaneous clinical remissions (Fig. 1), with negative Ham's tests in all nine who were tested. We later obtained blood samples from five of these nine patients; in all five the erythrocytes expressed normal levels of glycosylphosphatidylinositol-linked proteins, with no demonstrable PNH-affected erythrocytes (Fig. 3A). In addition, of the four patients tested, none had PNH-affected neutrophils (Fig. 3A), but three of the four had small numbers of PNH-affected lymphocytes (Fig. 3B). Further analysis by double fluorescent staining demonstrated a subpopulation of PNH-affected lymphocytes among CD4+

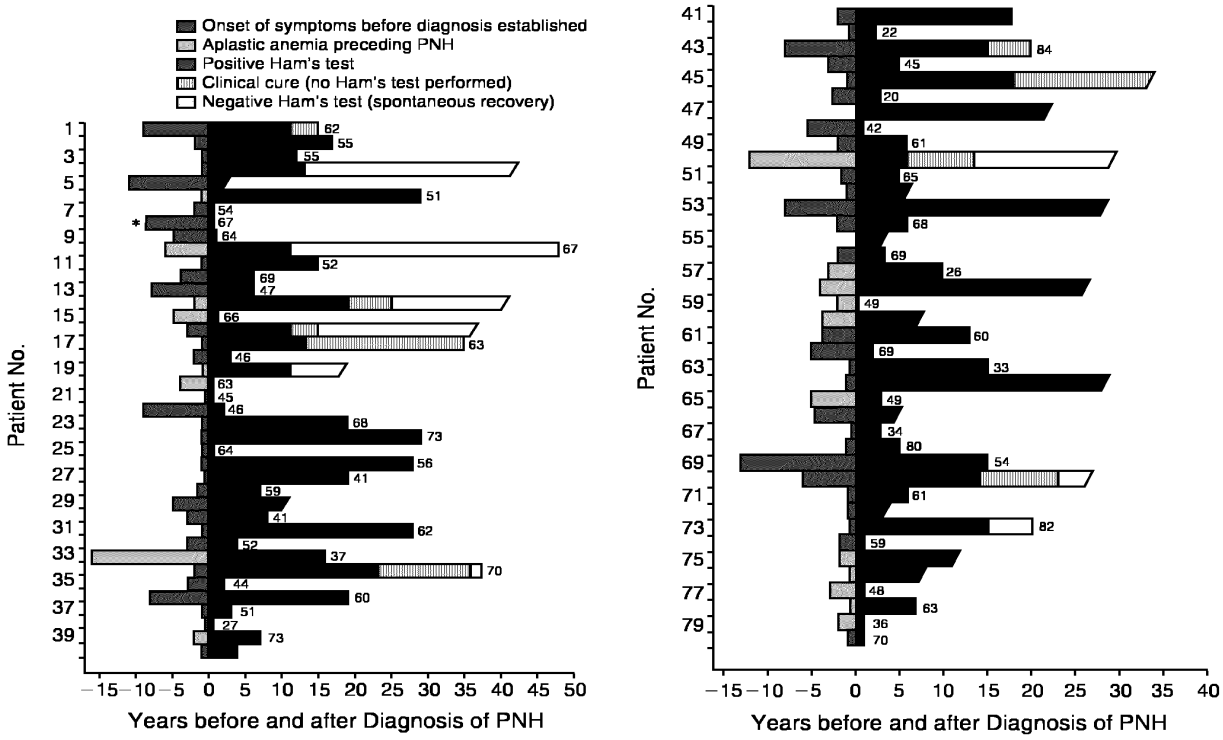


Figure 1. Course of Illness in 80 Consecutive Patients with PNH.

The x axis indicates the years before and after the diagnosis of PNH, which is denoted by the zero on the axis. Bars ending in a straight line indicate patients who died, and their ages at death are shown; bars ending in a diagonal line indicate surviving patients. Nine patients had complete clinical recovery, with negative Ham's tests. Three additional patients had protracted clinical remissions, but Ham's test was not repeated during remission. The asterisk indicates a prolonged but unspecified period of symptoms before the diagnosis was established.

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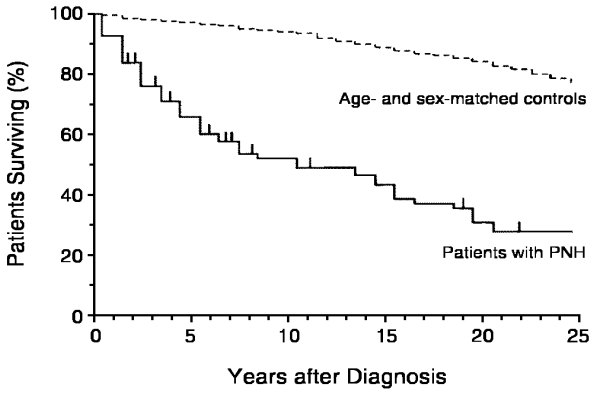


Figure 2. Actuarial Survival from the Time of Diagnosis in 80 Patients with PNH.

The median survival was 10 years. The expected survival of an age- and sex-matched control group is shown for comparison.

T cells, CD8+ T cells, and CD19+ B cells in all three patients.

Associated Disorders

Twenty-three patients (29 percent) received a diagnosis of aplastic anemia before the diagnosis of PNH. Nine of these patients subsequently had hemolytic PNH, with episodes of overt hemoglobinuria, and six did not; whether the remaining eight patients ever had hemoglobinuria could not be reliably determined.

At the time of the diagnosis, 64 of the 80 patients (80 percent) had thrombocytopenia (platelet count, <150,000 per cubic millimeter), and 38 patients (48 percent) had severe thrombocytopenia (platelet count, <50,000 per cubic millimeter). In addition, 44 patients (55 percent) had neutropenia (neutrophil count, <1500 per cubic millimeter).

In five patients the disorder progressed from hemolytic PNH to aplasia. Ham's test remained positive in four of these patients, but the fifth (Patient 34) subsequently had a negative Ham's test, with no PNH-affected erythrocytes or neutrophils. The patient died from bleeding due to severe thrombocytopenia 37 years after the diagnosis of PNH and at least 8 years after Ham's test had become negative.

Leukemia was not known to have developed in any of the patients.

Discussion

Clinical Features

In this group of 80 patients who were followed for up to 48 years, the median actuarial survival was 10 years, with 28 percent of the patients surviving for 25 years. Death was directly attributable to PNH or to bone marrow hypoplasia in 58 percent of the patients who died. Therapy with platelet concentrates should reduce mortality from hemorrhage due to thrombocytopenia.

At least 39 percent of the patients had venous thrombosis at some time during their illness. Many patients had initial thromboses that were life-threatening, but two (Patients 17 and 70), who probably had postpartum hepatic-vein thrombosis, recovered and survived for 34 and 27 years, respectively, after the thrombosis. One other patient (Patient 34) survived for 24 years after a thrombosis of the inferior mesenteric vein.

Spontaneous Remission

In some patients with PNH, the severity of the illness diminishes with time,[25] and some patients have a complete clinical remission,[22,26] although laboratory abnormalities may persist for years.[27] In this series 15 percent of the patients had spontaneous clinical remissions, and Ham's test became negative in the patients who were tested. Neither the severity of symptoms or complications nor the proportion of red cells lysed in Ham's test was correlated with an eventual remission. Among the patients with remissions, one (Patient 14) had severe, transfusion-dependent PNH and thromboses, and at

Table 1. Causes of Death in 60 Patients with PNH.

Cause of Death	No. of Patients
Probably related to PNH	
Venous thrombosis	
Hepatic vein	7
Inferior vena cava	1
Cerebral vein	1
Mesenteric vein	2
Pulmonary embolism	3
Hemorrhage	
Gastrointestinal	6
Subarachnoid	3
Intracerebral	2
Miscellaneous	
Liver failure	2
Intraabdominal event	1
Probably unrelated to PNH	
Arterial thrombosis	
Myocardial infarction	6
Cerebrovascular accident	2
Bronchopneumonia	3
Chronic obstructive airways disease plus cor pulmonale	2
Cardiac tamponade	1
Constrictive pericarditis	1
Renal failure	1
Amyloidosis	1
Lymphoma	1
Bronchial carcinoma	1
Epilepsy	1
Unknown	12

Table 2. Sites and Types of Thrombosis.

Site and Type of Thrombosis	No. of Patients
Intraabdominal	
Hepatic vein	8
Inferior vena cava	3
Mesenteric vein	4
Splenic vein	1
Renal vein	1
Unspecified	1
Other venous sites	
Cerebral vein	4
Pulmonary embolism	9
Deep vein	7
Superficial	3
Arterial	
Myocardial infarction	6
Cerebrovascular accident	2

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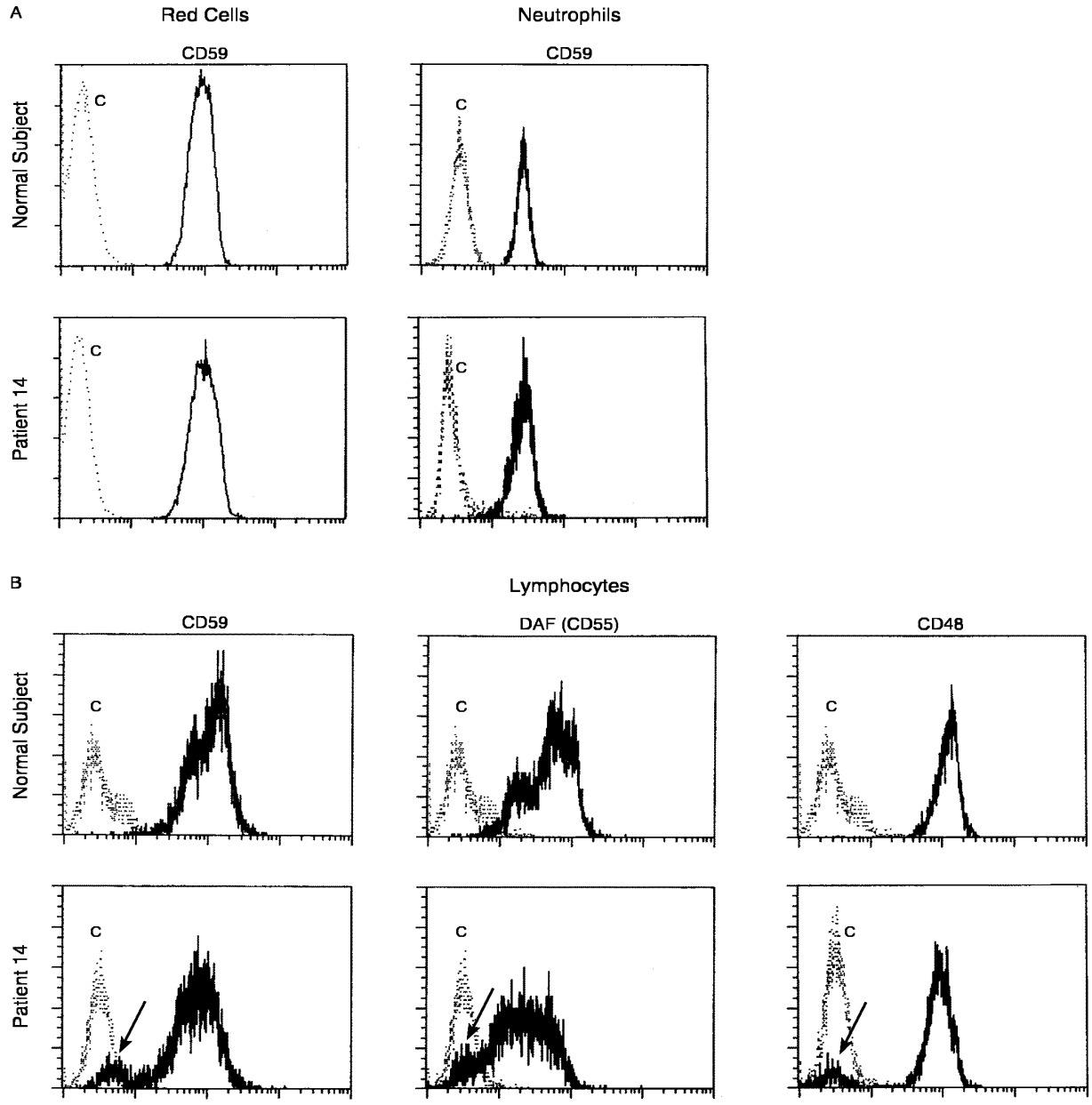


Figure 3. Flow-Cytometric Analysis of Blood Cells from a Normal Subject and Patient 14 after Spontaneous Recovery from PNH.

The analysis was carried out 20 years after the patient's clinical remission began and 17 years after her Ham's test had become negative. Panel A shows a unimodal distribution of cells with normal reactivity after reaction with anti-CD59 antibody (a molecule absent from the surface of PNH-affected cells). The distributions obtained with the patient's red cells and neutrophils are indistinguishable from those of the cells from a normal subject, demonstrating that no PNH-affected cells remain. C indicates distributions obtained in control experiments with an antibody directed against a molecule absent from normal blood cells.

Panel B shows the results of an analysis, similar to that in Panel A, carried out on peripheral-blood lymphocytes. The distributions of cells from a normal subject show that, as compared with the results of analyses with negative control antibody (C), all the lymphocytes reacted with all three antibodies used. The distribution is unimodal for CD48 and bimodal for CD59 and CD55. In contrast, the cells from Patient 14 have a small population of lymphocytes that are unreactive with all three antibodies (arrows), which indicates that these cells lack the corresponding antigen protein molecules on their surfaces. The fact that there is a peak of antigen-negative cells for three different proteins that are normally attached to the cell membrane by a glycosylphosphatidylinositol anchor means that the anchor is absent, which is characteristic of the PNH phenotype. The finding that the PNH clone was still present when there were no PNH-affected erythroid or myeloid cells in Patient 14 during a clinical and hematologic remission is consistent with the notion that this clone does not have a selective advantage once normal hematopoiesis has resumed. DAF denotes decay-accelerating factor.

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least two had over 50 percent lysis in earlier Ham's tests, suggesting that they had few residual normal stem cells at the height of their illness. The spontaneous remissions occurred between 10 and 20 years after the diagnosis of PNH. Of the 35 patients who survived 10 years or more, 12 (34 percent) eventually had remissions. A possible explanation for spontaneous remission is that the clones of PNH-affected cells have a finite life span, like normal somatic cells. Recovery may thus depend on the presence of normal stem cells capable of repopulating the bone marrow.[28]

There were no erythrocytes or neutrophils affected by PNH after recovery, but three of four patients tested had some residual abnormal lymphocytes. These results are consistent with the fact that lymphocytes have a much longer life span than myeloid and erythroid cells and that committed lymphocyte progenitor cells undergo division less frequently than the other cells. If the aging process postulated as being responsible for the gradual disappearance of clones of PNH-affected cells is related to the number of cell divisions, this may explain why abnormal lymphocytes persist for many years after the disappearance of abnormal neutrophils and erythrocytes.

PNH and Bone Marrow Failure

The data on this series of patients provide further evidence of the close relation between PNH and aplastic anemia. The coexistence of the two conditions in one patient was first reported in 1944,[29] with subsequent reports in 1952[30] and 1961.[31] Cultures of peripheral blood and bone marrow from patients with PNH consistently show reduced numbers of erythroid progenitor cells (erythroid burst-forming units) and myeloid progenitor cells (granulocyte–macrophage colony-forming units), even in the absence of pancytopenia,[32,33] as in patients with aplastic anemia.[34] In addition, PNH eventually develops in 10 to 31 percent of patients with aplastic anemia treated with immunosuppressive agents.[35] It is important to emphasize that PNH is not a complication of immunosuppressive treatment. Instead, the majority of patients with this disorder probably have an underlying aplastic process. Clones of PNH-affected cells may thus have a relative growth or survival advantage over the residual normal bone marrow in patients who have aplastic anemia.[1,28,36] It is conceivable that the mutation leading to the development of PNH occurs quite frequently but that, in the absence of marrow hypoplasia, the clone has difficulty establishing itself.[28]

PNH and Leukemia

Because of the reports of myelodysplasia and leukemia[1] — invariably, acute myeloid leukemia — in patients with PNH, it has been regarded as a preleukemic condition. Leukemia did not develop in any of our 80 patients, however, indicating that it is relatively rare in unselected patients with PNH. Acute myeloid leukemia eventually develops in approximately 5 percent of patients with aplastic anemia who survive the marrow aplasia.[35] Thus, aplastic anemia may predispose patients to both PNH and acute myeloid leukemia, but the development of PNH may not add to the risk of leukemia associated with uncomplicated aplastic anemia.

In most reported cases of leukemia in patients with PNH in whom the leukemic cells were studied,[37,38] the leukemic clone was derived from the PNH clone. According to a recent report of a patient with PNH who subsequently had a myelodysplastic syndrome, the blasts expressed normal levels of glycosylphosphatidylinositol-linked proteins,[39] indicating that the leukemic event may occur in the residual normal stem cells.

Management

The natural history of PNH is an important factor in decisions about therapy for individual patients. Thrombolytic treatment of hepatic-vein thrombosis with tissue plasminogen activator has been reported to be effective.[40] Oral anticoagulation therapy after venous thrombosis has been used since the 1950s.[25] In view of the high incidence of potentially fatal thrombosis, a strong case can be made for prophylactic anticoagulation treatment in all patients with PNH in whom there is no contraindication (such as severe thrombocytopenia). This approach may improve survival and reduce morbidity.

The only curative therapy at present is bone marrow transplantation, but it is available to only a small proportion of patients and is associated with substantial morbidity and mortality. The recent advances in the understanding of the pathogenesis of PNH and, in particular, of the molecular lesion open opportunities to explore new treatments, such as gene therapy. The possibility of spontaneous remission and of long-term survival must be taken into account when considering either bone marrow transplantation or other new treatments for this disorder.

We are indebted to the late Miss Eleanor Lloyd for her assistance in this project, to the patients, and to the physicians who originally referred the patients and provided follow-up data.

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Reference	Application No./Patent No.
AMG17203OP OPPO 01	11001632.6 - 1112 / 2359834
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
- ☐ Communication of
- ☒ Oral Proceedings on 21.01.2018

Enclosure(s):
- ☒ Letter from the proprietor of the patent of 21.11.2018
- ☒ Letter from the opponent 02 of 20.11.2018
- ☒ Copy (copies) P: claims AR 1,2, cited document D39-D41
  O2: cited documents D39, D40

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Our Ref.:	**198 441 u6/m25/ne** **Eculizumab Composition / ALEXION**
Opposition to:	**EP 2 359 834 B1 (11001632.6)**
Proprietor:	**Alexion Pharmaceuticals, Inc.** **Cheshire, CT 06410 (US)**
Opponent:	**Hoffmann Eitle** **Patent- und Rechtsanwälte PartmbB** **Arabellastr. 30** **81925 Munich (DE)**

The following observations are made in response to the preliminary opinion of the Opposition Division enclosed with their summons to oral proceedings issued on July 5, 2018.

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Page 7651

1. REFORMULATION OF TECHNICAL PROBLEM AND NEW EXPERIMENTAL DATA ARE NOT ADMISSIBLE

(1) The parties have agreed that **D1** represents the closest prior art. The OD sees no reasons to differ (cf. point 9 of the preliminary opinion). Starting from **D1**, the proprietor has reformulated the technical problem which is solved by the teaching of the opposed patent to be

> *"the ideal balance of properties for use in the treatment of PNH, including the **enhanced stability** and thus maintenance of biological integrity following prolonged storage in the absence of preservative, whilst also providing a low-volume liquid formulation having acceptable infusion time and which is convenient to store."*
> (cf. submission of March 22, 2018; point 5.11.1; emphasis added)

(2) To demonstrate that the above technical problem is solved, the proprietor provides post-published experimental evidence by Dr. Miano (**D33**). In the proprietor's opinion, the experiments of **D33** would show that the eculizumab formulation at 10 mg/ml is sufficiently stable for a period of 48 months. Notably, **D33** is only concerned with long term stability of the eculizumab preparation. No data were provided to support the other alleged benefits / advantages, such as "*maintenance of biological integrity*" or an "*acceptable infusion time*".

(3) It is undisputed that the original specification contains no disclosure at all with respect to the stability of the claimed eculizumab formulation, let alone that this aspect is mentioned or merely suggested anywhere.

(4) Still, the proprietor is of the opinion that the new stability data can be used as a basis to reformulate the objective technical problem. Specifically, he points to case law (as laid down in section G-VII-11 of the Guidelines and **T184/82**), according to which it is possible to rely on new effects, provided that the skilled person would recognize

Samsung Bioepis Exhibit No. 1002
Page 7652

these effects as implied by or related to the problem initially suggested. We disagree for the following reasons:

(5) The sole emphasis of the original disclosure was on the improvement of "*the quality of life of a patient*" and "*prolonging the health-adjusted life expectancy or a patient"* suffering from PNH" (cf. [0006] to [0010] of the opposed patent). Said "quality of life" and its measurement is defined in more detail in sections [0044] to [0052]. All the examples exclusively deal with clinical efficacy in the context of the improving of the patient's "*quality of life*".

(6) The proprietor now argues that the technical problem solved by the invention is to be seen in the enhancement of stability of the eculizumab formulation. We submit that this technical significance concerning the allegedly improved stability of a liquid eculizumab formulation is **not derivable** from the statements on quality of life of the patients made in the patent application. Moreover, even in the proprietor's opinion, this effect is not self-evident: he himself has indicated that the increased long-term stability was surprising.

(7) Further guidance with regard the derivability of a new technical effect or technical problem is provided by the established case law of the EPO. For example, in **T386/89** the board found that where a specific problem is identified in the description, the patentee may can put forward a

> *"**modified** version of the problem particularly if the issue of inventiveness has to be considered on an objective basis against a **new prior art** which **comes closer to the invention than that considered in the original patent application or granted patent specification**".*

(8) Reference is also made in this respect to the decision **T184/82** where the Board allowed a re-definition of the problem to such an extent that the skilled person

Samsung Bioepis Exhibit No. 1002
Page 7653

"*could recognise the same as implied or related to the problem initially suggested*" (cf. point 5 of the reasons).

(9) The opposition division will appreciate that **D1** is *de facto* **no** new prior art, as it would have been required by the above case law to allow reformulation of the technical problem. As outlined in our initial opposition brief (cf. point 3.2.2), **D1** basically comprises the same disclosure as **Hillmen et al**., N Engl. J Med **2004**; 350: 552-559 **(D3)**, i.e. the latter document could likewise have served as the closest prior art. **D3** is a scientific publication corresponding to the clinical study mentioned in the Examples of **D1** and describes the details of the procedure and the results of the study in more detail.

(10) Said **Hillmen 2004** reference is cited in the description of the opposed patent (cf. **ref. 12** on page 16; 4th document in left hand column on page 31). Referring to **D3,** it is stated in [0003]:

> *"In a preliminary, 12-week, open-label clinical study in 11 PNH patients, eculizumab was shown to reduce intravascular hemolysis and transfusion requirements.[12] However, this unblinded study involved a small number of patients with no control arm and without protocol-driven transfusion standards."*

(11) Section [0003] is immediately followed by the chapter "Summary of the Invention". This makes it clear that the inventors have not only carefully considered **D1/D3**, but also acknowledged it as the closest prior art document. Accordingly, they have formulated the technical problem to overcome potential deficiencies of the prior art study, such as a small number of patients, no control arm, and no protocol-driven transfusion standards. All this concerns "***quality of life***" of the patients, but not at all stability of the eculizumab formulation. Only with unallowable hindsight one could assume that improvement of long-term stability should be the "objective" technical problem to be solved by the claimed teaching.

Samsung Bioepis Exhibit No. 1002
Page 7654

(12) According to the above decisions, only if the technical the problem can be clearly deduced by the skilled person from the application as originally filed when considered in relation to the nearest prior art, reformulation of the problem would not contravene **Art. 123(2) EPC**, that is to say would not constitute added subject-matter.

(13) In this regard it is noteworthy that the whole TRIUMPH study underlying the opposed patent only lasted about **6 months** (cf. [0005], [0070], referring to a "*26 week treatment period*"). The proprietor now relies on a technical effect which – if at all – may only be visible **and** technically relevant **after two or three years** of storage of the formulation. Hence it would not have objectively been possible to identify the present technical problem of long-term stability from the present application, let alone from the closest prior art.

(14) Applying the above principles, we submit that the alleged unexpected effect, i.e. the allegedly improved long-term stability, is not deducible from the application as originally filed. It follows that it cannot be taken into account when determining the problem underlying the invention for the purpose of assessing the issue of inventive step. The post-published data of **D33** should not be admitted into the proceedings.

2. LACK OF INVENTIVE STEP (ARTICLES 100(A) AND 56 EPC)

(15) It is maintained that claim 1 of the opposed patent is obvious over **D1** (or **D3**) in combination with **D11,** or common general knowledge as reflected by **D10**.

2.1 D33 MERELY CONFIRMS COMMON KNOWLEDGE OF SKILLED PERSON BEFORE THE FILING DATE

(16) In **D33**, Dr. Miano provides data, allegedly demonstrating the improved stability of an eculizumab formulation at 10 mg/ml compared to 30 mg/ml. As outlined above, the new experiments should not be admitted into the proceedings and therefore not be

Samsung Bioepis Exhibit No. 1002
Page 7655

taken into account for the assessment of inventive step. However, even if the Opposition Division considers the data relevant, they are not sufficient to support inventive step. To explain in more detail:

(17) It was common general knowledge before the filing date of the opposed patent that increasing the concentration of antibodies increases the aggregation tendency of the protein, and thus decreases its stability. In fact, there is an almost linear relationship between an increase in concentration and aggregate formation. This is reflected, e.g., by the review article **D20**:

> *"Increasing the concentration of antibodies often increases the aggregation tendency of the protein. It was demonstrated that increasing the IgG1 concentration (in 10 mM citric acid, 100 mM NaCl pH 5.5) from 2.7 mg/mL to 50 mg/mL almost linearly increased the Nephelometric Unit (NU) from 2 to 40.[52]"* (p. 9, left col., 3rd para. of **D20**)

(18) Hence, it was entirely predictable that an antibody composition formulated at 10 mg/ml shows less protein aggregation (i.e. a higher monomer content) than a composition formulated at 30 mg/ml. The data presented in **D33** merely confirm this trivial fact, which would have been obvious to the skilled person before the filing date of the opposed patent.

(19) In fact, the data submitted with **D33** illustrate the "***try-and-see***" approach the Proprietor has taken to find a concentration of 10 mg/ml. According to Dr. Miano, *"one of the stability parameters that the commercial formulation of eculizumab must meet in order to be deemed satisfactorily stable and thus suitable for release into the market is the percent of monomer antibody in the sample must be greater than or equal to 98.0%, which means that there must be less than or equal to 2.0% aggregates of the antibody […]"* (Item 3 of **D33**). In his submission, the Proprietor confirms that at least 98% monomer content represents a commercial requirement (cf. Item 5.3.4 of the Proprietor's submission dated March 22, 2018).

Samsung Bioepis Exhibit No. 1002
Page 7656

(20) Having the commercial requirement of 98% monomer content in mind, and knowing that aggregate formation of antibodies increases with increasing concentration, it was a routine task for the skilled person to test a variety of antibody concentrations, and determine the storage time after which the monomer content reaches the threshold level of 98%. **D35** shows that antibody concentrations between 0.5 mg/ml and 50 mg/ml were routinely used for pharmaceutical formulations before the filing date of the opposed patent, and thus would have been included in the stability tests conducted by the skilled person.

(21) In **D33**, Dr. Miano describes the exact kind of stability studies that would have been routine to the skilled person. However, eculizumab formulations at merely two (standard) antibody concentrations were used, i.e. at 10 and 30 mg/ml. For Figure 1, these formulations were incubated at 23-27°C, in order to simulate the degradation that would occur over an extended period of time at lower temperatures. For Figure 2, the formulations were incubated at 2-8°C, which is the recommended storage temperature for eculizumab. The monomer content was assessed at various time points. However, in the experiment conducted at 2-8°C the last time point for the concentration of 10 mg/ml was taken after 24 months, whereas for the concentration of 30 mg/ml the last time point was taken after only 18 months. Any information relating to later time points is only based on extrapolation, and not on actual data, as confirmed by Dr. Miano (cf. last sentence of Item 7 of **D33**).

(22) Notably, in both of Figures 1 and 2 of **D33**, only single data points for each time point are shown for the formulation at 30 mg/ml, suggesting that only a single batch was analysed. This is contrary to the international guidelines for stability testing of biological product, which recommend providing stability information on at least 3 batches (cf. Item 4.3 of **D39**), and casts serious doubts on the reliability of any of the data for a concentration of 30 mg/ml. **D39** is filed in direct response to the Proprietor's new technical arguments regarding the stability of the claimed eculizumab composition. Thus, should the Proprietor's new technical arguments be

Samsung Bioepis Exhibit No. 1002
Page 7657

admitted into the proceedings (which we object to; cf. Item 1 above), also **D39** should be admitted.

(23) Additionally, from Dr. Miano's statements in **D33** and the presented data it appears that the data for the 10 mg/ml and 30 mg/ml samples were not generated in side-by-side experiments. In Item 4 of **D33**, Dr. Miano indicates that a 30 mg/ml formulation was generated, but there is no mentioned of preparing a 10 mg/ml formulation. Consistently, according to **D33**, only the batch of 30 mg/ml (designated "ALXN5100") was subjected to the experimental procedure described in Item 5. It thus appears that only the data for the 30 mg/ml samples were generated specifically for **D33**. The source of the data for the 10 mg/ml samples remains elusive – they may have been generated for governmental approval of the 10 mg/ml eculizumab formulation. The notion that the 10 mg/ml and the 30 mg/ml samples have not been tested in side-by-side experiments could also explain the experimental differences outlined above, i.e. that only a single batch was tested for the 30 mg/ml concentration vs. several batches for the 10 mg/ml concentration, and that the samples at 10 mg/ml and 30 mg/ml were tested for different periods of time in Figure 2.

(24) If the data presented in **D33** were indeed not generated in side-by-side experiments, no reliable conclusions can be drawn from them. This is because it is known that various external (e.g., environmental) factors can significantly influence the results of biological experiments. These external factors differ from day to day. When experiments are then carried out over many months, as in the present case, the influence of these external factors multiplies, and severely distorts the experimental results. Merely to name one example, the temperatures at which the experiments of Figures 1 and 2 were performed are given as ranges, i.e. 23-27°C and 2-8°C, respectively. A high external temperature is likely to have shifted the actual temperature at which the experiments were performed close to the upper limit, and *vice versa*. Hence, it is entirely possible that the experiments for the 30 mg/ml samples were generated at a temperature close to 8°C, and the experiments for the

Samsung Bioepis Exhibit No. 1002
Page 7658

10 mg/ml samples at close to 2°C. Of course, such differences would have a significant impact on degradation, which is accelerated at high temperatures. For the above reasons, no reliable conclusions can be drawn from the stability studies presented in **D33**.

(25) If the data presented in **D33** are considered despite the above-described deficiencies, Figure 2 suggests that at a temperature of 2-8°C and a concentration of 30 mg/ml, the tested eculizumab formulation reached the threshold level of 98% monomer content after about 36 months of storage. Dr. Miano concludes that *"the 30 mg/ml concentration would not meet the release standards after 36 months of storage"* (Item 5.3.7 of the Proprietor's submission dated March 22, 2018). At a concentration of 10 mg/ml, the monomer content of the eculizumab formulation after 36 months of storage was about 99.5%, and thereby above the threshold level of 98%. Accordingly, a concentration of 10 mg/ml was found acceptable.

(26) However, as mentioned above, already before the filing date of the opposed patent it was common general knowledge that aggregate formation of antibodies increases almost linearly with increasing concentration (p. 9, left col., 3rd para. of **D20**). Hence, based on Figure 2, any antibody concentration between 30 mg/ml and 10 mg/ml would have a monomer content of at least 98% after 36 months of storage, and would thus be acceptable for market release. In fact, if it was desired to obtain eculizumab compositions that are even more stable than the one formulated at 10 mg/ml, the antibody concentration could simply be reduced to below 10 mg/ml. Apparently to disguise from these trivial facts, no comparative data of eculizumab compositions formulated at any other concentrations, e.g. at 20 mg/ml or below 10 mg/ml are included in **D33**.

(27) Overall, the above demonstrates that the skilled person was in a classical "try and see" situation when finding a suitable concentration for formulating eculizumab. According to established case law, a "try and see" situation is considered to have

occurred if the skilled person, in view of the teaching in the prior art, had already clearly envisaged a group of compounds or a compound (here: standard concentrations) and then determined by routine tests whether such compound/s (here: concentrations) had the desired effect (cf. I.D.7.2 of the EPO Case Law of the Boards of Appeal).

(28) For example, in the case underlying **T1241/03**, the board considered the problem underlying the patent in suit to be the provision of an aqueous formulation of human growth hormone which remains stable during long-term storage. The Proprietor argued that that a skilled person, knowing from the prior art that the development of stable aqueous protein formulations was a risky business, would not have had a reasonable expectation of success to arrive at the claimed subject-matter upon combination of the teaching in two prior art documents. However, the board pointed out that a reasonable expectation of success does not require certainty. Thus, the board held that in spite of the understandable uncertainties which always characterise experiments using biologic compounds like proteins, the skilled person had no reason to adopt a sceptical attitude. He or she would have had either some expectations of success or, at worst, no particular expectations of any sort, but only a "try and see" attitude, which does not equate with the absence of an reasonable expectation of success (cf. Item 32 of the “Reasons for the Decision” of **T1241/03**). Accordingly, the board denied an inventive step.

(29) The present case is comparable to the case underlying **T1241/03**. However, in the present case the skilled person did not even have to combine the teachings of different prior art documents. Rather, the skilled person could simply rely and his or her common general knowledge that aggregate formation of antibodies increases with increasing concentrations. Accordingly, faced with the problem of providing a stable eculizumab formulation, he or she would have tested a number of standard concentrations and would have found that concentrations lower than 30 mg/ml, e.g. 10 mg/ml, provide for the desired stability. Just like in **T1241/03**, the skilled person

Samsung Bioepis Exhibit No. 1002
Page 7660

had no reason to be sceptical that one of the tested antibody concentrations would solve the problem of providing a stable eculizumab formulation, i.e. a formulation with a monomer content of at least 98% after 36 months of storage at 2-8°C.

(30) Additionally, it is important to consider that no particular advantage of an eculizumab concentration of 10 mg/ml has been demonstrated by the Proprietor. As outlined above, based on Figure 2 any antibody concentration between 30 mg/ml and 10 mg/ml would have a monomer content of at least 98% after 36 months of storage, and would thus be acceptable for market release. Thus, the claimed antibody concentration of 10 mg/ml eculizumab represents an entirely arbitrary selection.

(31) Overall, faced with the problem of providing a stable eculizumab formulation, the skilled person was in a classic "try and see" situation. He or she would have conducted standard stability studies, such as the ones presented in **D33**. The data shown in **D33** are unreliable but, if anything, they merely confirm the skilled person's expectation that formulations are more stable at low antibody concentrations than at high concentrations. In accordance with **D33**, when performing standard stability studies the skilled person would have realized that any antibody concentration lower than 30 mg/ml would be acceptable for market release. The specific concentration of 10 mg/ml represents an arbitrary selection which is not associated with any unexpected technical effect. It is established case law of the EPO that a technical effect can only corroborate the patentability of an invention if it is unexpected or surprising. Thus, even if considered, the data of **D33** do not support an inventive step.

2.2 10 MG/ML IS A TYPICAL ANTIBODY CONCENTRATION ALSO FOR NON-IGG1 ANTIBODIES

(32) In his submission dated March 22, 2018, the Proprietor argues that based on the presented evidence, e.g. **D20**, it cannot be concluded that 10 mg/ml represents a

Samsung Bioepis Exhibit No. 1002
Page 7661

typical antibody concentration in pharmaceutical formulations. To support his arguments, the Proprietor refers to **D35**, in which he presents an overview of all antibodies listed in **D20** and **D21**. Strikingly, even according to **D35**, which was prepared by the Proprietor himself, the concentration of 10 mg/ml represents the single most frequent concentration among all of the listed antibody solutions. Hence, the evidence presented by the Proprietor himself supports the view that 10 mg/ml represents a typical antibody concentration in pharmaceutical formulations, which the skilled person certainly would have considered also for eculizumab formulations.

(33) In **D35**, the Proprietor has additionally included information on the isotypes of the listed antibodies. According to **D35**, eculizumab is an IgG2/4 antibody, whereas most of the other antibodies included in the list are of the IgG1 isotype. IgG2/4 antibodies were known before the filing date of the opposed patent, as evidenced e.g. by **D40**. However, the Proprietor alleges that due to the structural differences between non-IgG1 and IgG1 antibodies, no conclusions for a suitable antibody concentration for eculizumab formulations can be drawn from prior art relating to IgG1 antibodies. Moreover, the Proprietor argues that none of the five non-IgG1 antibodies in **D35** were formulated at a concentration of 10 mg/ml. Based on these observations, the Proprietor alleges that, before the filing date of the opposed patent, a concentration of 10 mg/ml was not typical for non-IgG1 antibodies.

(34) We refute the Proprietor's allegation that a concentration of 10 mg/ml was not typical for pharmaceutical non-IgG1 antibody formulations before the filing date of the opposed patent. To support our view, we herewith submit prior art documents **D41** to **D43**, all of which demonstrate that a concentration of 10 mg/ml was also common for non-IgG1 antibodies. **D40** to **D43** are filed in direct response to the Proprietor's allegation that no conclusions for a suitable antibody concentration of non-IgG1 antibodies can be drawn from prior art relating to IgG1 antibodies. Therefore, the documents should be admitted into the proceedings.

Samsung Bioepis Exhibit No. 1002
Page 7662

(35) Additionally, it should be considered that the opposed patent does not mention any significance of the antibody isotype for the stability of eculizumab formulations. The only mention of isotypes in the opposed patent is found in paragraph [0055], and relates to the recognition of IgG1 and IgG3 antibodies by phagocytic cells. The specific isotype of eculizumab (IgG2/4) is not even mentioned in the opposed patent. Hence, **D40** to **D43** are filed in response to an unpredictable technical argument by the Proprietor, and should be admitted into the proceedings also for this reason.

(36) **D41** relates to the pharmaceutical IgG4 antibody natalizumab. The particular focus of **D41** is to provide stable antibody formulations:

> *"Therefore, notwithstanding what has previously been reported in the literature, there exists a need for improved methods of formulating proteins and/or antibodies. There is also a need for stable formulations with large concentrations of antibody or protein, where the activity of the antibody or protein is retained. Also needed are stable formulations of concentrated protein which maintain a fixed volume. Applicants disclose herein stable compositions which can be further utilized to prepare antibody formulations, especially formulations with high concentrations of antibody which do not precipitate out and are stable when stored at the recommended temperatures. The highly concentrated and stable antibody formulations will greatly aid physicians in treating subjects of variable weights."* (p. 1, ll. 25-34 of **D41**)

(37) In **D41** the problem of providing a stable natalizumab composition is solved by adding phosphate buffer, polysorbate and sodium chloride. That way, pharmaceutical formulations comprising antibody concentrations between 0.1 and 200 mg/ml can be prepared (cf. p. 2, ll. 1-9 of **D41**). The concentration of 10 mg/ml is singled out on page 13, lines 2 to 5 of **D41**. Notably, Examples 11 and 12 list all ingredients of two exemplary, stable natalizumab formulations at exactly 10 mg/ml (pp. 23-24 of **D41**). Like the eculizumab composition of the opposed patent, the natalizumab compositions of **D41** are preservative-free. These data show that a

Samsung Bioepis Exhibit No. 1002
Page 7663

concentration of 10 mg/ml is a typical concentration also for non-IgG1 antibody formulations, e.g. for formulations comprising IgG4 antibodies such as natalizumab.

(38) **D42** relates to the IgG4 antibody volociximab (p. 2, ll. 6-8 and p. 8, l. 22 of **D42**). The antibody targets the α5β1 integrin and inhibits the growth of cancer cells (cf. p. 1, ll. 5-7 of **D42**). On page 20, lines 19 to 23, **D42** indicates that in a preferred embodiment, the antibody is formulated as a pharmaceutical composition comprising a solution of between about 1.0 and 15.0 mg/ml antibody. **D42** further states that *"in a particularly preferred embodiment the pharmaceutical composition comprises a solution of about 10.0 mg/mL antibody, […]"* (p. 20, ll. 24-26 of **D42**). In a table listing the parameters of a phase II clinical study, the concentration of volociximab compositions for treating patients with metastatic renal cell carcinoma is indicated to be 10 mg/ml (cf. p. 41, "Dosage Form and Strength/Formulation" of **D42**). Hence, also **D42** demonstrates that a concentration of 10 mg/ml is a typical concentration for non-IgG1 antibody formulations.

(39) Finally, **D43** relates to antibodies that regulate the activity of NK cells. Although the preferred antibody is of the IgG4 isotype, reference is also made to IgG1, IgG2 and IgG3 antibodies (cf. claims 15-16 of **D43**). The preferred pharmaceutical composition comprising these antibodies has a concentration of 10 mg/ml:

> *"For example, an antibody present in a pharmaceutical composition of this invention can be supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials."* (p. 73, ll. 17-19 of **D43**)

(40) Overall, **D35** as presented by the Proprietor himself in combination with **D41**, **D42** and **D43** show that, before the filing date of the opposed patent, a concentration of 10 mg/ml was not only typical for pharmaceutical formulations of IgG1 antibodies, but also common for non-IgG1 antibodies such as IgG4. In view thereof, the skilled

Samsung Bioepis Exhibit No. 1002
Page 7664

person would certainly have considered a concentration of 10 mg/ml for the IgG2/4 antibody eculizumab, e.g. when performing stability studies as outlined above.

3. CONCLUSION

(41) The data contained in **D33** should not be admitted into the proceedings. However, even if admitted, they do not support an inventive step. Moreover, before the filing date of the opposed patent, formulating antibody compositions at 10 mg/ml was common in the field, also for non-IgG1 antibodies. Thus, as laid out in our notice of opposition dated August 9, 2017, all of the features in combination as claimed in the opposed patent were obvious over **D1** (or **D3**) in combination with **D11,** or common general knowledge as reflected by **D10**.

(42) Consequently, the opposed patent does not meet the requirements of the EPC. It has therefore to be revoked *in toto*.

(43) We will use the English language in the Oral Proceedings on January 21, 2019. A translation from English into German is not required. Should any other language besides English be used by any party, translation into English is required.



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Samsung Bioepis Exhibit No. 1002
Page 7665

Enclosures:

D39 ICH Harmonised Tripartite Guideline on Stability Testing of Biological Products

D40 Mueller et al. (1997): Humanized porcine VCAM-specific monoclonal antibodies with chimeric IgG2/G4 constant regions block human leukocyte binding to porcine endothelial cells. Mol Immunol. 34(6): 441-452.

D41 WO 2004/071439 A2

D42 WO 2005/092073 A2

D43 WO 2006/003179 A2

Samsung Bioepis Exhibit No. 1002
Page 7666

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HUMANIZED PORCINE VCAM-SPECIFIC MONOCLONAL ANTIBODIES WITH CHIMERIC IgG2/G4 CONSTANT REGIONS BLOCK HUMAN LEUKOCYTE BINDING TO PORCINE ENDOTHELIAL CELLS

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Abstract—Discordant xenografts surviving the initial hyperacute rejection phase may be subject to cellular rejection processes mediated by infiltrating leukocytes including T cells, NK cells and monocytes. The stable adhesion of these cell types to endothelial cells is due to the molecular interaction of the integrins VLA-4 and LFA-1 with their ligands vascular cell adhesion molecule (VCAM) and ICAM-1 present on the endothelial cells. Human VLA-4 binds to porcine VCAM, and blocking mAbs specific for porcine VCAM have been developed. We have localized the epitope of the anti-porcine VCAM blocking mAbs 2A2 and 3F4 to domains 1 and 2, respectively. Humanized antibodies (IgG4 isotype) were constructed from these anti-porcine VCAM antibodies and demonstrated to inhibit adhesion of Ramos, Jurkat and YT cells, as well as purified resting and activated human T cells, to porcine aortic endothelial cells (PAEC). These cell types express both LFA-1 as well as VLA-4, suggesting blockade of human VLA-4 interaction with porcine VCAM may alone be sufficient to significantly impair adhesion of human leukocytes to porcine endothelial cells. The chimeric anti-porcine VCAM (pVCAM) HuG4 antibodies promoted increased adhesion of Fc receptor (FcR) positive cells such as U937 monocytic cells to PAEC. In contrast, chimeric anti-porcine VCAM antibodies created using the CH1 and hinge region from human IgG2 and the CH2 and CH3 regions from human IgG4 (HuG2/G4 antibodies) inhibited binding of FcR positive cells to PAEC. These chimeric anti-pVCAM antibodies should allow delineation of the *in vivo* role of VLA-4/VCAM interaction in porcine-to-primate xenotransplants. Further, the design of the HuG2/G4 antibodies should render them efficacious in multiple settings requiring elimination of FcR binding. © 1997 Elsevier Science Ltd.

Key words: adhesion molecules, antibodies, Fc receptors, inflammation, xenotransplantation.

INTRODUCTION

Although much progress has been made in elucidating the molecular mechanisms of hyperacute rejection of xenotransplants (reviewed in Somerville and D'Apice, 1993), the mechanisms of cell mediated rejection of xenografts remain unclear. The cellular infiltrate observed 3 days following transplantation of guinea pig hearts into complement (C)-depleted rats is comprised predominantly of macrophages and natural killer cells, with low numbers of T lymphocytes present (Blakely *et al.*, 1994). Similarly, the cellular infiltrate present 6 days after transplantation of porcine fetal islet clusters into rats is comprised predominantly of macrophages with low numbers of T lymphocytes present primarily at the periphery of the graft (Wallgren *et al.*, 1995). *In vitro* porcine aortic endothelial cells (PAEC) as well as antigen presenting peripheral blood cells can directly activate human CD4+ and CD8+ T lymphocytes (Rollins *et al.*, 1994*a*; Murray *et al.*, 1994; Yamada *et al.*, 1995). Thus the initial phases of cellular rejection of xenotransplants are likely to involve binding and activation of multiple types of leukocytes including not only T lymphocytes but also natural killer cells and monocytes.

The stable adhesion of lymphocytes, natural killer cells, and monocytes to endothelial cells is mediated predominantly by the interaction of the integrins LFA-1 (CD11a/CD18) and VLA-4 (CD49d/CD29) with their cognate ligands ICAM-1 and VCAM-1 on endothelial cells (Allavena *et al.*, 1991; Jonjic *et al.*, 1992). For both monocytes (Luscinskas *et al.*, 1994) and T lymphocytes (Alon *et al.*, 1995; Luscinskas *et al.*, 1995) the binding of VLA-4 to VCAM is an essential component of the *in vitro* adhesive interactions required for stable arrest under

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Samsung Bioepis Exhibit No. 1002
Page 7667

conditions of flow. *In vitro*, the transmigration of NK cells and monocytes across human umbilical vein endothelial cell (HUVEC) monolayers also utilizes both LFA-1/ICAM-1- and VLA-4/VCAM-dependent pathways (Bianchi *et al.*, 1993; Meershaert and Furie, 1994; Chuluyan *et al.*, 1995). *In vivo*, the interaction of LFA-1 with ICAM-1 may not be completely essential for extravasation of lymphocytes or monocytes since both of these cell types are present in inflammatory infiltrates of leukocyte adhesion deficiency patients who have a genetic deficiency in CD18 and thus lack integrins such as LFA-1 (Hawkins *et al.*, 1992). Finally, numerous studies have demonstrated the potential of blocking antibodies directed against LFA-1 (Issekutz, 1992; Scheynius *et al.*, 1993), VLA-4 (Issekutz, 1991; Yednock *et al.*, 1992; Chisholm *et al.*, 1993), ICAM-1 (Zeng *et al.*, 1994), or VCAM (Orosz *et al.*, 1993; Silber *et al.*, 1995) to inhibit the accumulation of lymphocytes at inflammatory sites in animals.

For xenogeneic cellular interactions it is not yet fully defined which adhesion molecule interactions will function across species barriers. It is likely that at least some xenogeneic receptor–ligand interactions will be nonfunctional. For example, mouse LFA-1 does not bind to human ICAM-1 (Johnston *et al.*, 1990). To explore the molecular basis of adhesive interactions between distinct classes of adhesion molecules on human and porcine cells we have cloned porcine E-selectin and VCAM-1 and have demonstrated that both are capable of mediating the adhesion of human leukocytes (Rollins *et al.*, 1994*a*; Mueller *et al.*, 1995). Further, we have developed murine monoclonal antibodies (mAbs) specific for porcine VCAM which block binding of human VLA-4 to porcine VCAM (Mueller *et al.*, 1995). The functional blocking activity of these antibodies suggests that they will be useful in suppressing the cellular response to a porcine xenograft. However, murine mAbs are not ideally suited for use in primates due to their short half-life and high immunogenicity (Khazaeli *et al.*, 1994). Here we have utilized antibody engineering strategies to create humanized anti-porcine VCAM antibodies that block adhesion of human natural killer cells, monocytes, and T lymphocytes to activated PAEC. Furthermore, we show the choice of human isotype has critical implications for the potential therapeutic use of humanized antibodies in xenotransplantation.

MATERIALS AND METHODS

Cells

Ramos, Jurkat and U-937 cells were obtained from the American Type Culture Collection, YT cells were the gift of Dr J. M. Wang (National Cancer Institute, Frederick, MD, U.S.A.), porcine aortic endothelial cells (PAEC) were obtained from Cell Systems (Kirkland, WA, U.S.A.) and 293-EBNA cells were obtained from Invitrogen (San Diego, CA, U.S.A.). U-937 cells were maintained in RPMI 1640 supplemented with 15% heat-inactivated FBS and 2 mM L-glutamine. Ramos and Jurkat cells were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 20 mM HEPES, 50 μM β-mercaptoethanol, 100 units/ml penicillin and 100 μg/ml streptomycin. YT were maintained in RPMI 1640 containing 10% heat-inactivated FBS, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. PAEC were maintained in medium A consisting of M199 supplemented with 20% heat-inactivated FBS, 0.05 mg/ml ECGF (Biomedical Technologies, Stoughton, MA, U.S.A.), 20 mM HEPES and 0.1 mg/ml heparin, and were used within three passages. 293-EBNA cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin and 250 μg/ml G418. Human T cells were purified as described previously (Mueller *et al.*, 1995).

Production of VCAM/Ig fusions

A plasmid containing the human IgG1 Fc region was obtained from Dr M. J. Lenardo (NIH, Bethesda, MD, U.S.A.). DNA fragments containing various domains of human or porcine VCAM were obtained by PCR amplification of appropriate templates and joined in frame to the human IgG1 Fc coding region using standard molecular biology techniques. The cassettes encoding the fusion proteins were ligated into the expression plasmid pAPEX-3 (Evans *et al.*, 1995*a*). VCAM/Ig fusion proteins were produced by transfection of 293-EBNA cells as described previously (Evans *et al.*, 1995*a*). Three days following transfection the media were harvested, centrifuged to remove debris, and stored at 4°C. For ELISA, PolySorp 96-well microtiter plates (Nunc, Naperville, IL, U.S.A.) were coated overnight at 4°C with 100 ng/well goat anti-human IgG Fc (Sigma, St. Louis, MO, U.S.A.) in 0.1 M carbonate buffer, pH 9.6. The plates were then washed three times with PBS supplemented with 0.5% Tween 20 and incubated with blocking buffer (PBS supplemented with 0.05% Tween 20) at 37°C for 1 hr. After three further washes, various dilutions of conditioned media in blocking buffer were added and incubated at 37°C for 1 hr. Following three further washes, 2A2 or 3F4 mAb in blocking buffer was added and incubated for 1 hr at 37°C. After additional washing, peroxidase-conjugated goat anti-mouse IgG Fc (Sigma) diluted 1:2000 in blocking buffer was added and incubated at 37°C for 1 hr. After three final washes the plate was developed for 15 min with 0.4 mg/ml *o*-phenylenediamine dihydrochloride (Sigma). Reactions were stopped by addition of 1 M sulfuric acid. Quantitation was performed by measuring absorbance at 490 nm using a Bio-Rad model 3550 plate reader.

Production of chimeric antibodies

Variable regions were cloned from the 2A2 and 3F4 hybridomas by PCR amplification as described previously (Evans *et al.*, 1995*a*, 1995*b*). These sequences have been submitted to the EMBL/GenBank/DDBL under accession numbers U78799–U78802. The variable regions were joined to genomic DNA cassettes containing

Samsung Bioepis Exhibit No. 1002
Page 7668

either the intact human IgG4 constant regions or the first constant region and hinge linker region from human IgG2 followed by the second and third constant regions from human IgG4 using standard molecular biology techniques. Chimeric antibodies were produced in 293-EBNA cells and purified by chromatography on protein A Sepharose as described (Evans *et al.*, 1995*a*). Each chimeric antibody preparation was greater than 95% pure as judged by Coomassie staining of SDS polyacrylamide gels. F(ab')2 was prepared by digestion of the antibodies with ficin (Pierce, Rockford, IL, U.S.A.) followed by protein A chromatography.

Flow cytometry

Cells used were obtained by trypsinization (PAEC and 293-EBNA) or centrifugation (Ramos, Jurkat, YT and U-937 cells). Harvested cells were washed once with 4°C PBS containing 1% heat-inactivated FBS (PBS/1) and aliquoted at 50 000–100 000 cells per well in 96-well V-bottom plates (Nunc). Cells were washed two additional times with PBS/1, and then incubated with PBS/1 containing 10 μg/ml primary Ab or PBS/1 alone for 30 min at 4°C. For some experiments the chimeric HuG4 or HuG2/G4 was centrifuged at $1 \times 10^5 g$ for 1 hr prior to addition to the cells. The cells were then washed twice with PBS/1 and resuspended in PBS/1 containing the appropriate FITC-conjugated secondary antibodies (1:200 dilution) for 30 min at 4°C. The cells were again washed twice with PBS/1 and resuspended in PBS. Cell fluorescence was determined using a Becton Dickinson FACSort.

Adhesion assays

On day 0 PAEC were plated in medium A at a density of 10 000 cells per well in a 96-well flat bottom plate. On day 1 the PAEC were refed medium A supplemented with 1 μg/ml LPS (Sigma, *E. coli* 0111:B4). On day 2, human cells were resuspended in the appropriate growth medium supplemented with 10 μM calcein AM (Molecular Probes, Eugene, OR, U.S.A.) and incubated at 37°C for 30 min. The cells were subsequently washed three times with growth medium and resuspended at 6×10^6 cells/ml in RPMI 1640 supplemented with 1% heat-inactivated FBS (RPMI/1). The PAEC were washed twice with RPMI/1 and refed 50 μl/well of RPMI/1 containing the indicated concentrations of antibodies. After adding calcein-labeled cells at a density of 3×10^5 cells/well (50 μl), the plate was centrifuged at 200*g* for 1 min and incubated at 37°C for 15 min. The nonadherent cells were removed by washing three to five times with RPMI/1. Adherent cells were lysed with HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm. Background fluorescence was determined from wells containing PAEC that did not receive calcein-labeled cells. Samples were performed in duplicate in each experiment. Adherence was determined by comparing the residual fluorescence concentration in each well to the input fluorescence concentration. In each experiment the specific fluorescence signal obtained in the absence of competing antibody was defined as 100%.

For adhesion assays using purified soluble pVCAM (Mueller *et al.*, 1995), PolySorp 96-well microtiter plates (Nunc) were coated overnight at 4°C with 200 ng/well purified soluble pVCAM in 0.1 M carbonate buffer, pH 9.6. After blocking the plate with RPMI 1640 supplemented with 10 mg/ml BSA (RPMI/BSA), the plates were incubated with RPMI/BSA containing antibody for 1 hr at 37°C. Subsequently, 3×10^5 calcein-labeled U-937 cells in RPMI/BSA were added to each well. The plate was centrifuged upright for 1 min at 200*g* and incubated at 37°C for 15 min. The plate was then centrifuged inverted at 200*g* for 3 min. Adherent cells were lysed by the addition of HBSS containing 1% SDS and calcein fluorescence was measured with a Millipore CytoFluor 2350 using an excitation wavelength of 485 nm and an emission wavelength of 530 nm.

RESULTS

Identification of porcine VCAM domains recognized by blocking mAbs

Previously, we have identified two murine mAbs (3F4 and 2A2) directed against porcine VCAM (pVCAM) which block VLA-4 dependent adhesion of peripheral blood human T cells to PAEC. These two mAbs are specific for pVCAM and do not recognize human VCAM (hVCAM). Here an initial analysis of the epitope specificity of the 2A2 and 3F4 mAbs was performed using fusion proteins consisting of various domains of pVCAM or hVCAM joined to the Fc region of human IgG1. The VCAM/Ig fusion proteins were produced in 293-EBNA cells and assayed for binding of 2A2 and 3F4 mAbs by ELISA (Fig. 1). Both the 2A2 and 3F4 mAbs bound efficiently to proteins containing the first three domains of pVCAM. The 2A2 mAb bound to fusion proteins containing pVCAM domain 1 such as pD1 + pD2 (porcine domain 1 plus porcine domain 2) and pD1 + hD2 (porcine domain 1 plus human domain 2), but did not bind to fusion proteins lacking pVCAM domain 1 such as pD2 + pD3, hD1 + pD2, or hD1 + hD2. Weak binding of the 2A2 mAb could also be detected to a fusion protein containing only porcine domain 1 (pD1) but not to fusion proteins containing only pD2 or pD3. Conversely, the 3F4 mAb bound to fusion proteins containing pVCAM domain 2, such as pD1 + pD2, pD2 + pD3, and hD1 + pD2, but did not bind to fusion proteins lacking pVCAM domain 2, such as pD1 + hD2 and hD1 + hD2. Binding of the 3F4 mAb could not be detected to fusion proteins containing only a single pVCAM domain. The weak binding of 2A2 and 3F4 to fusion proteins containing only a single VCAM domain is similar to previously characterized blocking antibodies specific for human VCAM and ICAM-1 which require the presence of at least two adhesion molecule domains for binding (Pepinsky *et al.*, 1992; Renz *et al.*, 1994). Together, these results suggested the 2A2 mAb specificity is defined by

Samsung Bioepis Exhibit No. 1002
Page 7669

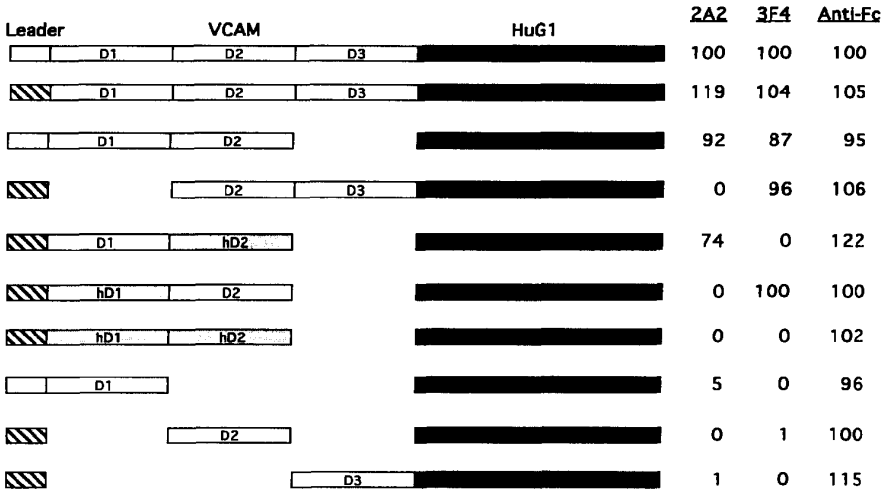


Fig. 1. Binding of 3F4 and 2A2 mAbs to VCAM/Ig fusion proteins. Proteins consisting of domains 1 (D1), 2 (D2), or 3 (D3) from porcine VCAM (open boxes) or human VCAM (gray boxes) joined to the Fc region of human IgG1 (black boxes) were produced in 293-EBNA cells. In some constructs the pVCAM secretory leader sequence was replaced with the secretory leader sequence from human CD59 (striped boxes). The fusion proteins were captured by incubating diluted conditioned media in 96-well plates coated with goat anti-human IgG (Fc specific). After incubation with 2A2 or 3F4 mAb, bound murine mAb was detected using peroxidase-conjugated goat anti-mouse IgG (Fc specific). The total amount of each fusion protein captured per well was quantitated using peroxidase-conjugated goat anti-human IgG (Fc specific). The absorbance signal obtained with the fusion protein containing the intact pVCAM domains 1, 2 and 3 was defined as 100% for each antibody. Binding of the 2A2 and 3F4 mAbs to the remaining fusion proteins is reported as a percentage of this value. Results shown are from a representative experiment of several performed.

pVCAM domain 1 whereas the 3F4 mAb specificity is defined by pVCAM domain 2.

Inhibition of human leukocyte binding to PAEC by chimeric anti-porcine VCAM mAbs

The use of murine antibodies in porcine-to-primate xenotransplantation models or ultimately in porcine-to-human xenotransplants poses several problems including a short half-life of the murine antibody plus development of an immune response directed against the murine antibody. These problems have been significantly reduced by the use of chimeric antibodies consisting of the murine variable regions joined to human constant regions (Khazaeli *et al.*, 1994). To create anti-pVCAM chimeric antibodies we cloned the variable regions from both 2A2 and 3F4. The murine variable heavy regions were joined to the human IgG4 constant region (HuG4) since this isotype does not activate complement (C) (Tao *et al.*, 1993), the predominant mediator of hyperacute rejection of xenografts. The murine variable light regions were joined to the human kappa constant region. Chimeric ch2A2 HuG4 and ch3F4 HuG4 antibodies were produced by coexpression of the chimeric heavy and light chains in 293-EBNA cells and tested for binding to pVCAM by FACS analysis using 293 cells expressing pVCAM (Fig. 2(A)). Both chimeric mAbs produced identical staining profiles as the corresponding murine mAbs, while neither chimeric mAb bound to untransfected 293 cells. Additionally, both the chimeric antibodies and murine mAbs produced identical FACS profiles using LPS-activated porcine aortic endothelial cells (Fig. 2(B)), confirming the chimeric antibodies retained the ability to bind to pVCAM expressed on PAEC.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to block the interaction between human VLA-4 and porcine VCAM was assessed by measuring inhibition of Ramos cell binding to activated PAEC (Fig. 3). The murine 3F4 mAb inhibited this binding by 90% when present at concentrations greater than 5 μg/ml. A control humanized antibody directed against human C5 (h5G1.1 HuG4) did not inhibit the binding of Ramos to activated PAEC. The ch2A2 HuG4 and ch3F4 HuG4 produced nearly identical inhibition curves as the murine 3F4 mAb, demonstrating the retention of binding avidity and cell adhesion blocking capabilities in these chimeric antibodies.

The ability of the ch2A2 HuG4 and ch3F4 HuG4 mAbs to inhibit binding of cells expressing both VLA-4 and LFA-1 was next examined using the Jurkat T cell line. Both the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of Jurkat to activated PAEC by up to 90% while the control antibody h5G1.1 HuG4 did not inhibit binding at any concentration (Fig. 4(A)). Similarly, the ch2A2 HuG4 and ch3F4 HuG4 antibodies inhibited the binding of both resting (Fig. 4(B)) and activated (Fig. 4(C)) T cells to activated PAEC, although the

Samsung Bioepis Exhibit No. 1002
Page 7670

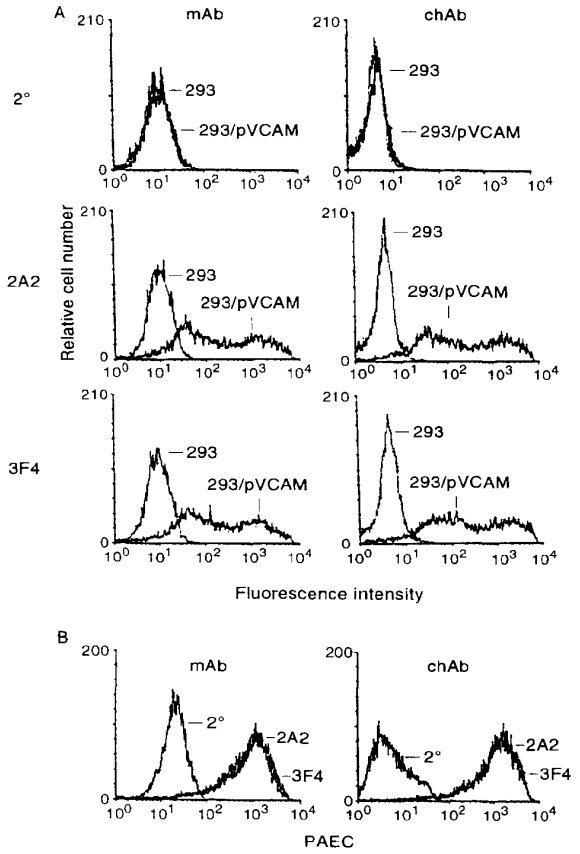


Fig. 2. Binding of 3F4 and 2A2 chimeric antibodies to pVCAM. (A) Chimeric mAbs containing the variable regions of either 2A2 or 3F4 joined to human IgG4 and kappa constant regions were produced in 293-EBNA cells and purified by protein G Sepharose chromatography. Untransfected 293-EBNA cells (293) or 293-EBNA cells expressing pVCAM (293/pVCAM) were incubated with 10 μg/ml purified murine 2A2 mAb, murine 3F4 mAb, ch2A2 HuG4, or ch3F4 HuG4. Bound antibody was detected using either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-human IgG followed by flow cytometry using a Becton Dickinson FACSort. Data are displayed as histograms with fluorescence intensity plotted on the x-axis and relative cell number plotted on the y-axis. Background staining by the secondary antibodies is shown in the panels labeled 2°. (B) PAEC were activated by treatment with 1 μg/ml LPS for approximately 16 hr. Cells were then stained with either the murine 2A2 and 3F4 mAbs (left panel) or the ch2A2 HuG4 and ch3F4 HuG4 antibodies (right panel). Background staining by the secondary antibodies (2°) is indicated.

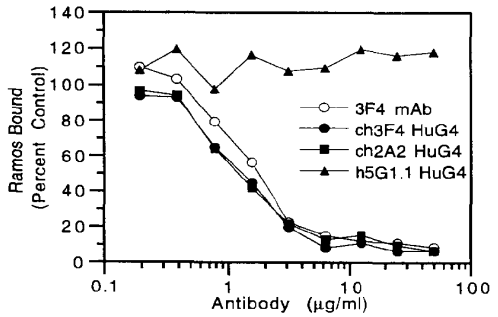


Fig. 3. Binding of Ramos cells to PAEC in the presence of mAbs. On day 0, PAEC were plated at 10 000 cells per well in medium A in 96-well plates. On day 1 the PAEC were activated by adding fresh medium A supplemented with 1 μg/ml LPS. After 16 to 24 hr incubation, the PAEC were washed and refed RPMI 1640 containing 1% FCS plus the indicated concentrations of the murine 3F4 mAb (3F4 mAb), the chimeric 2A2 and 3F4 mAbs (ch2A2 HuG4 and ch3F4 HuG4) or a humanized negative control antibody (h5G1.1 HuG4). Calcein-labeled Ramos cells were added and allowed to adhere to the PAEC under static conditions for 15 min. After nonadherent cells were removed by washing, the remaining bound cells were lysed with SDS. Calcein fluorescence of the SDS supernatant was measured with a Millipore CytoFluor 2350. In each experiment the binding of Ramos cells to PAEC in the absence of antibody was defined as 100%. Results shown are the mean of two to four experiments, with each value determined in duplicate in each experiment.

maximal inhibition achieved was reduced to approximately 60%. Addition of a blocking mAb directed against human VLA-4 also maximally inhibited adhesion by 60% (Fig. 4(C)), confirming the binding inhibition observed was due to blockade of the molecular interaction between human VLA-4 and porcine VCAM.

The potential of the ch2A2 HuG4 and ch3F4 HuG4 antibodies to inhibit binding of natural killer cells and monocytes to activated PAEC was examined using the cell lines YT (Yodio *et al.*, 1985) and U-937 (Sundstron and Nilsson, 1976), respectively. Binding of YT cells to activated PAEC was strongly inhibited by concentrations of 10 μg/ml ch2A2 HuG4 and ch3F4 HuG4 antibodies (Fig. 5(A)). In contrast, the ch2A2 HuG4 and ch3F4 HuG4 antibodies did not inhibit binding of U-937 cells to activated PAEC (Fig. 5(B)). One possible explanation for this failure to inhibit U-937 cell binding could be the presence of elevated levels of VLA-4 on U-937 cells. However, FACS analysis revealed the presence of comparable levels of CD49d and activated CD29 on U-937 cells as on other cell lines such as Jurkat whose adhesion to PAEC was inhibited by the chimeric anti-pVCAM antibodies (Fig. 6). Interestingly, the adhesion of U-937 to PAEC was consistently augmented by approximately 50% in all experiments. This increase was specific for the anti-pVCAM antibodies as no increase in U-937 binding to PAEC was detected in the presence of the control h5G1.1 HuG4 antibody (Fig. 5(B)). This suggested that binding of U-937 to PAEC could be increased through FcR binding to the anti-pVCAM HuG4 antibodies on the PAEC surface. In support of this hypothesis, FACS analysis confirmed the presence of significant levels of FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) on the surface of U-937 cells. In contrast, Jurkat and YT cells had no detectable Fcγ receptors while Ramos cells showed the presence of only low levels of FcγRII (Fig. 6).

Binding of the HuG4 chimeric antibodies to U-937 could be directly demonstrated by flow cytometry (Fig.

Samsung Bioepis Exhibit No. 1002
Page 7671

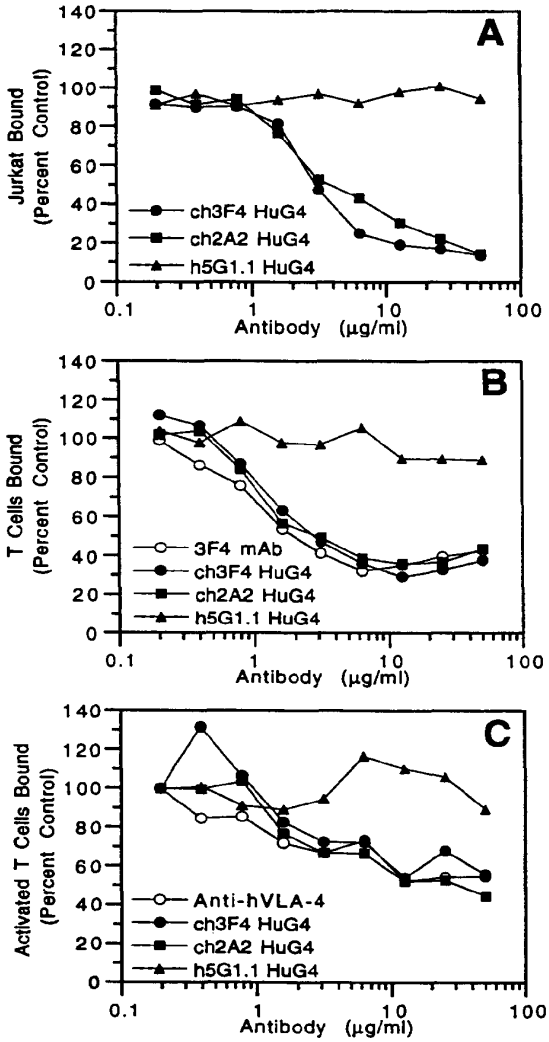


Fig. 4. Binding of Jurkat and human T cells to PAEC in the presence of HuG4 antibodies. Binding experiments were performed as described in Fig. 3 using calcein-labeled Jurkat cells (A) or calcein-labeled purified human T cells (B and C). Human T cells were either assayed immediately after purification (B) or cultured in the presence of 1 μg/ml PHA for 5 days followed by 1 day without PHA stimulation (C) prior to performing the adhesion assay. Results shown are the mean of two to four experiments (A and B) or from an individual experiment (C). Anti-hVLA-4 denotes the blocking murine mAb HP2/1 directed against the α4 subunit of human VLA-4.

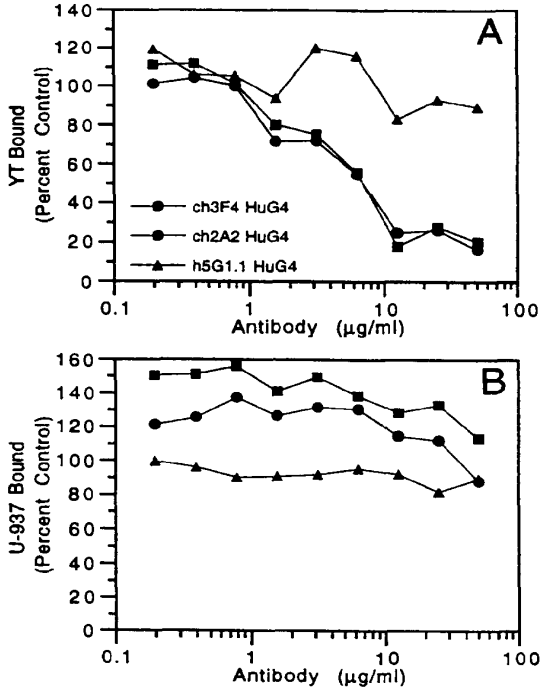


Fig. 5. Binding of YT and U-937 to PAEC in the presence of HuG4 mAbs. Adherence of calcein-labeled YT cells (A) or U-937 cells (B) to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

7). Both the HuG4 antibodies as well as the anti-human C5 HuG4 antibody bound to U-937 cells. In contrast, 293/pVCAM cells bound only the anti-pVCAM antibodies but not the anti-human C5 HuG4 antibody. No binding of the HuG4 chimeric antibodies could be detected with Ramos, Jurkat, or YT cells. These results suggest that the HuG4 chimeric antibodies bound to U-937 cells through interaction of the HuG4 Fc region with the Fc receptors (FcRs) present on U-937 cells. Finally, F(ab')2 fragments prepared from ch3F4 HuG4 strongly inhibited binding of U-937 cells to PAEC (Fig. 8) whereas F(ab')2 fragments prepared from h5G1.1 HuG4 did not inhibit the binding. This result demonstrates that, if antibody crosslinking is prevented, blockade of the interaction between human VLA-4 and porcine VCAM can effectively inhibit the adhesion of human monocytes to PAEC.

Inhibition of FcR binding of humanized mAbs with altered constant regions

Although F(ab')2 fragments proved capable of inhibiting adhesion of U-937 cells, antibody fragments are suboptimal for chronic treatments, as they have much shorter *in vivo* half-lives than intact antibodies (Covell *et al.*, 1986). The use of intact antibodies with human constant regions as anti-rejection agents is complicated by the capacity of such antibodies to fix C and/or to bind FcRs. Human antibodies of the G4 isotype are devoid of C activity (Tao *et al.*, 1993; Smith *et al.*, 1995). Human antibodies of the G2 isotype do not activate C or bind FcRs (Canfield and Morrison, 1991; Burton and Woof, 1992). Rather than altering the specific residues known to mediate C fixation and FcR binding, we explored the possibility of creating an antibody with a composite human constant region. To create chimeric mAbs which would not activate C or bind to FcRs, we utilized the overall similarity in the human G2 and G4 primary

Samsung Bioepis Exhibit No. 1002
Page 7672

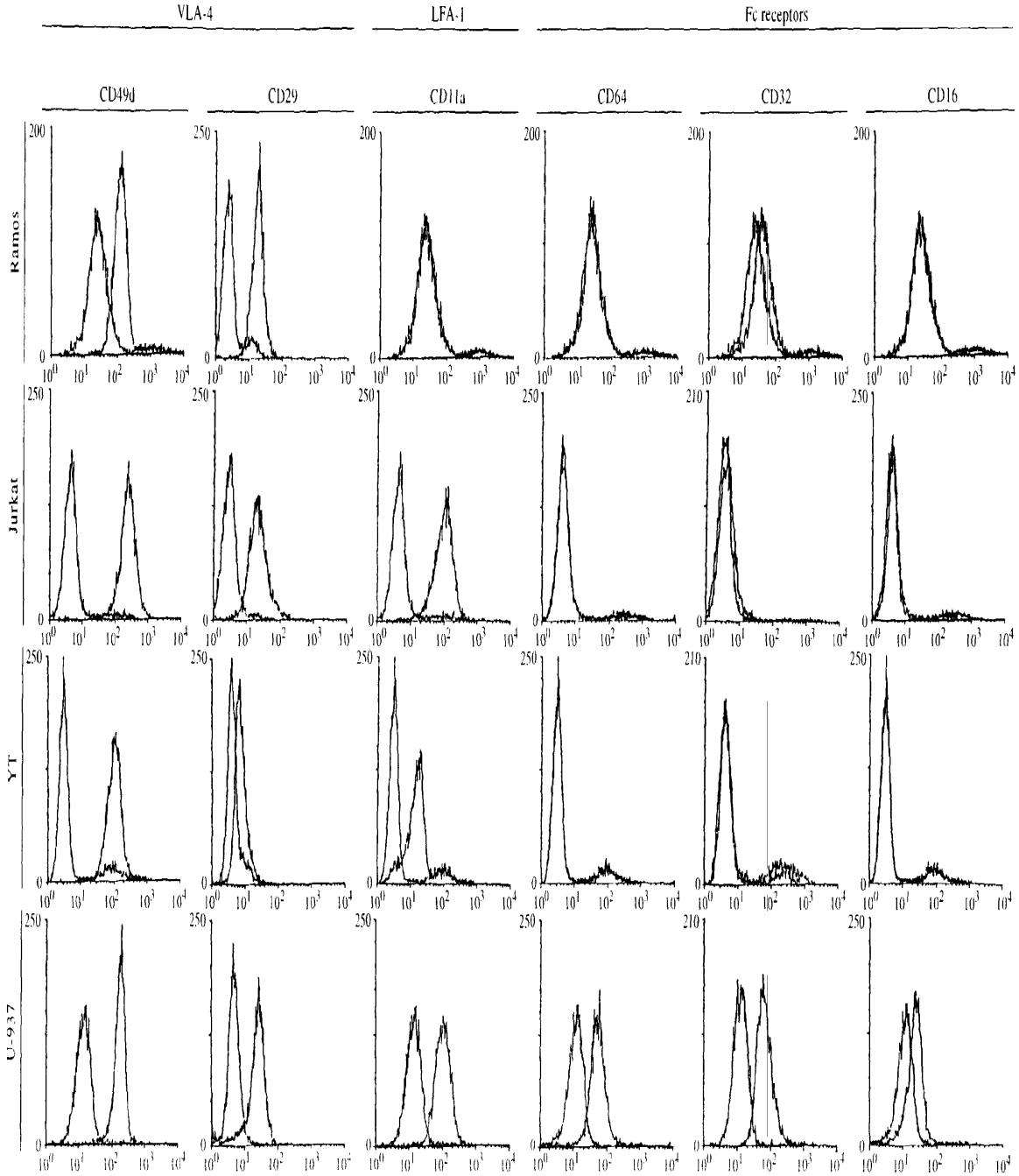


Fig. 6. Expression of VLA-4, LFA-1 and FcRs on the surface of Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml of the following primary antibodies: mouse anti-human CD49d, rat anti-activated human CD29, mouse anti-human CD11a, mouse anti-human CD64 ($Fc_\gamma RI$), mouse anti-human CD32 ($Fc_\gamma RII$) or mouse anti-human CD16 ($Fc_\gamma RIII$). After washing, cells were incubated with either FITC-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-rat IgG as appropriate. Each panel presents the results obtained in the presence of primary plus secondary antibody or in the presence of secondary antibody alone.

Samsung Bioepis Exhibit No. 1002
Page 7673

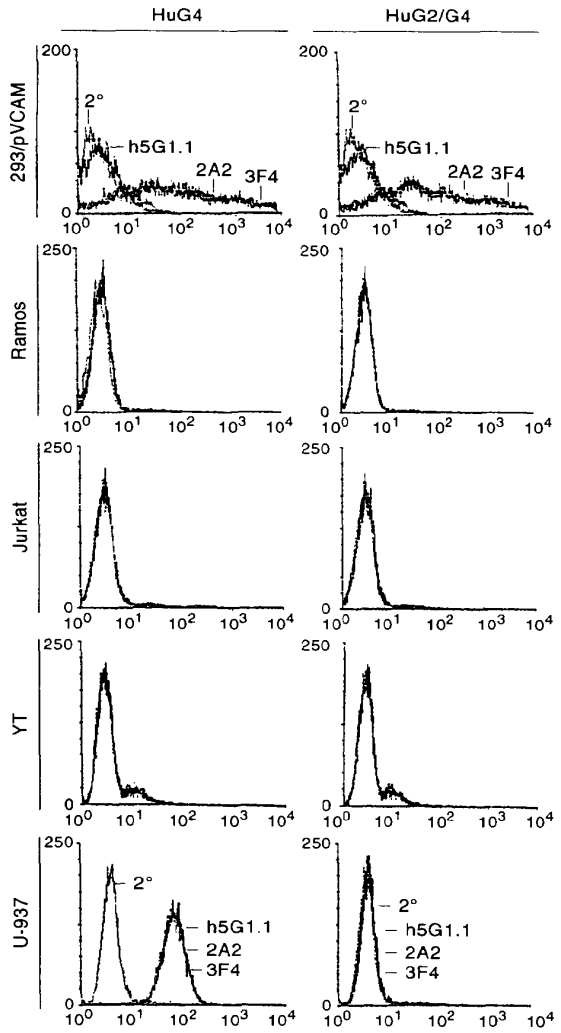


Fig. 7. Binding of chimeric antibodies to 293/pVCAM, Ramos, Jurkat, YT and U-937 cells. Cells were incubated with 10 μg/ml h5G1.1 HuG4, ch2A2 HuG4, or ch3F4 HuG4 (left panels) or 10 μg/ml h5G1.1 HuG2/G4, ch2A2 HuG2/G4, or ch3F4 HuG2/G4 (right panels). After washing, cells were incubated with FITC-conjugated goat anti-human IgG except for Ramos cells which were incubated with FITC-conjugated goat anti-human IgG (γ chain specific). Each panel presents the results obtained with the indicated cell line for h5G1.1 HuG4, ch2A2 HuG4 and ch3F4 HuG4 mAbs as well as the secondary antibody alone (2°) control.

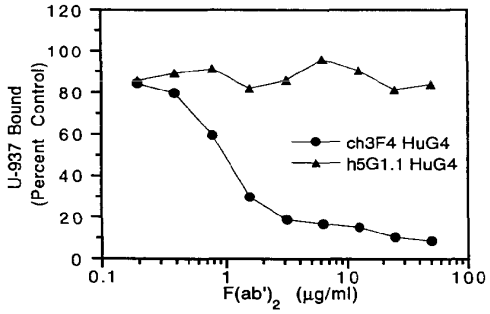


Fig. 8. Binding of U-937 cells to PAEC in the presence of F(ab')2 fragments. F(ab')2 fragments were prepared from h5G1.1 HuG4 and ch3F4 HuG4 by digestion with ficin. Undigested antibody and Fc fragments were removed by protein A chromatography. Adherence of calcein-labeled U-937 cells to PAEC in the presence of F(ab')2 was assayed as described in Fig. 3. Results shown are the mean of five experiments.

sequences to design a HuG2/G4 composite antibody consisting of the first constant region domain and the hinge region from the HuG2 isotype joined to the second and third constant region domains from the HuG4 isotype (Fig. 9). The resulting mAb did not contain the antibody sequences necessary for FcR binding, nor any new epitopes that would likely be immunogenic.

Binding to FcR was examined by using the monocytic cell line U937. FACS analysis demonstrated that the resulting ch3F4 HuG2/G4, ch2A2 HuG2/G4 and h5G1.1 HuG2/G4 antibodies did not bind to U-937 cells (Fig. 7). The ch3F4 HuG2/G4 and ch2A2 HuG2/G4 antibodies maintained the ability to bind pVCAM as demonstrated both by binding to 293/pVCAM cells (Fig. 7) and by inhibition of Jurkat and YT binding to activated PAEC (Fig. 10). For both Jurkat and YT cells inhibition of binding with the chimeric HuG2/G4 was equivalent to the HuG4 or original murine anti-pVCAM antibodies. The binding of U-937 cells to PAEC was now potently inhibited by the presence of either ch2A2 HuG2/G4 or ch3F4 HuG2/G4 antibodies. Finally, the chimeric antibodies were examined for the ability to block the adhesion of U-937 cells to purified soluble pVCAM (Fig. 11). Addition of the ch3F4 HuG4 antibody failed to inhibit binding of U-937 cells to pVCAM unless very high concentrations of antibody were added. In contrast the ch3F4 HuG2/G4 antibody potently inhibited the binding of U-937 cells to pVCAM, while the negative control h5G1.1 HuG2/G4 antibody did not block binding. Together, these results demonstrate the anti-pVCAM HuG2/G4 antibodies specifically inhibited the adhesive interaction of human VLA-4 with porcine VCAM and that disruption of this interaction was sufficient to inhibit binding of numerous human cell types to porcine endothelial cells.

DISCUSSION

Current success in allotransplantation has resulted from the development of effective global immunosuppressive therapies that prevent cellular graft rejection. Recent attempts to develop more specific immunosuppressive strategies have focused on inhibiting interactions between integrins and costimulatory molecules on T cells with their ligands on graft endothelial cells and other antigen presenting cells (reviewed in Faustman, 1995; Guinan *et al.*, 1994). The success of allotransplantation has been limited by the resulting shortage of human donor organs. Recent progress in overcoming the initial

Samsung Bioepis Exhibit No. 1002
Page 7674

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HuG2      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG2/G4   STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV
HuG4      STKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGV

HuG2      HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG2/G4   HTFPAVLQSSGLYSLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVE
HuG4      HTFPAVLQSSGLYSLSSVVTVPSSSLGTKTYTCNVDHKPSNTKVDKRVE

                                235
HuG2      RKCCVECPPCPAPPV- AGPSVFLF
HuG2/G4   RKCCVECPPCPAPPV- AGPSVFLF
HuG4      SKYGPPCPSCPAPEFLGGPSVFLF

HuG2      PPKPKDTLMISRTPEVTCVVVDVSHEDPEVQFNWYVDGVEVHNAKTKPR
HuG2/G4   PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR
HuG4      PPKPKDTLMISRTPEVTCVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPR

                                                      331
HuG2      EEQFNSTFRVVSVLTVVHQDWLNGKEYKCKVSNKGLPAPIEKTISKTKG
HuG2/G4   EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
HuG4      EEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKG
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Fig. 9. Sequence of the CH1, hinge and CH2 regions of human IgG2 (HuG2), human IgG4 (HuG4), and the chimeric HuG2/G4 construct. Positions where differences occur between the three molecules are double underlined. The amino acid sequence at position 235/236 determines ability to bind to Fcγ receptors (Canfield and Morrison, 1991; Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) while the presence of a serine at position 331 confers the inability to activate C (Tao *et al.*, 1993).

hyperacute rejection of discordant xenografts suggests the potential for use of porcine xenografts as a clinical option to circumvent this chronic shortage. Although there are limited data regarding the fate of discordant xenografts which survive the initial hyperacute rejection phase, xenograft recipients will likely require continued cellular immunosuppression for the prevention of graft rejection. In this light, xenografts offer a unique opportunity for the development of blocking reagents specific for the graft, thereby avoiding global immunosuppression of the recipient.

To investigate the interactions occurring between human leukocytes and porcine endothelial cells, we have cloned the porcine VCAM molecule and created mAbs specific for porcine VCAM that do not bind to human VCAM (Mueller *et al.*, 1995). Two of these antibodies (3F4 and 2A2) are capable of blocking the interaction between human VLA-4 and porcine VCAM. Here we demonstrate that the binding specificity for 2A2 is located within domain 1 of pVCAM while the binding specificity for 3F4 is located within domain 2. This is identical to the results found with human VCAM, where blocking antibodies have also been shown to be directed against either domains 1 or 2 (Osborn *et al.*, 1992; Vonderheide and Springer, 1992).

The use of murine mAbs in humans and primates presents numerous problems including a short antibody half-life and a high degree of immunogenicity (Khazaeli *et al.*, 1994). To optimize the use of the anti-porcine VCAM antibodies in porcine-to-primate xenograft experiments we developed chimeric antibodies using the variable regions cloned from the 2A2 and 3F4 antibodies joined to the constant regions of the human IgG4,κ isotype. Given the extensive activation of C which occurs within xenografts, the human IgG4 isotype was chosen due to its inability to activate C. The resultant chimeric antibodies (ch2A2 HuG4 and ch3F4 HuG4 antibodies) retained specificity for porcine VCAM and were as potent as the original murine mAbs at blocking adhesion of Ramos cells to activated PAEC by greater than 90% (Fig. 3). In contrast to Ramos, the binding of resting or activated human T cells to activated PAEC was inhibited only approximately 50% by either the chimeric antibodies or the original mAbs (Fig. 4). Activation of human T cells by PAEC can be blocked by antibodies directed against either CD2 or LFA-1 (Rollins *et al.*, 1994*b*), suggesting either of these molecules could mediate the remaining adhesion by interacting with porcine CD58 or porcine ICAM-1, respectively.

The adhesion of human lymphocytes, monocytes or NK cells to activated HUVEC is dependent on the interaction of both LFA-1 with ICAM-1 and VLA-4 with VCAM (Allavena *et al.*, 1991; Jonjic *et al.*, 1992; Vonderheide and Springer, 1992). For each cell type, blocking the VLA-4/VCAM interaction by itself produces only a small reduction in adhesion, while antibodies which disrupt the LFA-1/ICAM-1 interaction provide a greater, although still partial, inhibition of adhesion. Addition of antibodies blocking both VLA-4/VCAM and LFA-1/ICAM-1 is required to completely block adhesion of these cell types to HUVEC. Surprisingly, binding of LFA-1$^+$–VLA$^+$4 Jurkat or YT cells to activated PAEC could be inhibited nearly completely by the ch3F4 HuG4 and ch2A2 HuG4 antibodies. One possible explanation for this could be the lack of interaction between human LFA-1 and porcine ICAM. The conservation of ICAM-1 is relatively low between species, with human and mouse ICAM-1 being only 50% conserved at the amino acid level (reviewed in Carlos and Harlan, 1994), and *in vitro* mouse LFA-1 does not bind to human ICAM-1. Alternatively, human LFA-1 may interact with porcine ICAM-1, but with a reduced affinity. For example, human VLA-4 binds to human VCAM at two independent sites, one within domain 1 and one within

Samsung Bioepis Exhibit No. 1002
Page 7675

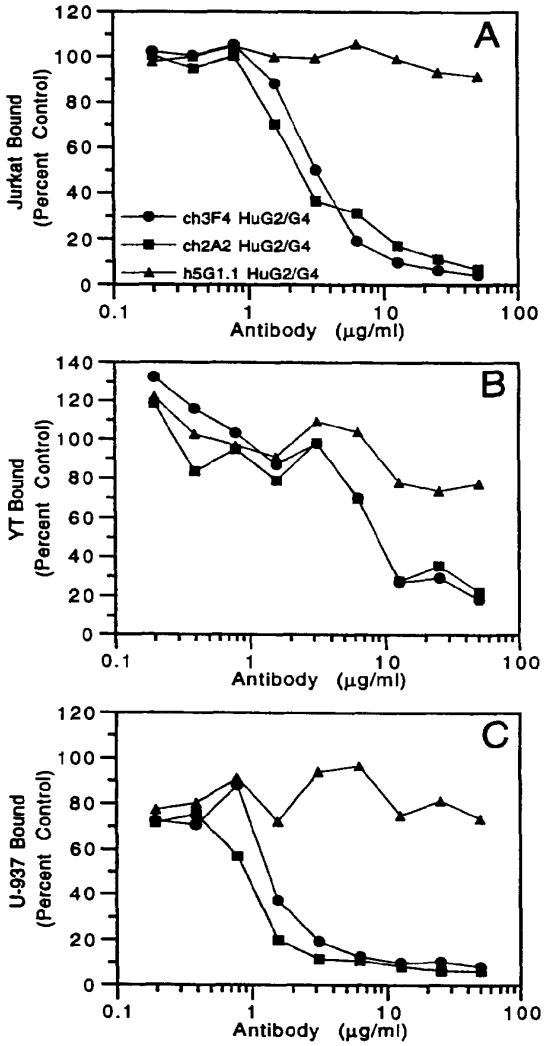


Fig. 10. Binding of Jurkat, YT and U-937 to PAEC in the presence of HuG2/G4 mAbs. Adherence of calcein-labeled Jurkat (A), YT (B) and U-937 (C) cells to PAEC was assayed as described in Fig. 3. Results shown are the mean of two to four experiments.

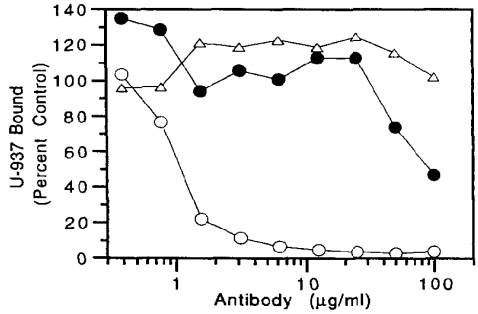


Fig. 11. Binding of U-937 to purified pVCAM. Microtiter plate wells were coated with purified soluble pVCAM. After blocking with FCS, wells received the indicated concentrations of either ch3F4 HuG4 (●), ch3F4 HuG2/G4 (○) or h5G1.1 HuG2/G4 (△) followed by 3×10^5 calcein-labeled U-937 cells. The cells were allowed to adhere at 37°C for 15 min after which non-adherent cells were removed by washing. Adherence of U-937 cells in the absence of competitor antibody was defined as 100%. Results shown are from an individual representative experiment.

domain 4 (Osborn *et al.*, 1992; Vonderheide *et al.*, 1994). In contrast, porcine VCAM lacks domain 4, suggesting the interaction between human VLA-4 and porcine VCAM may be weaker than the interaction between human VLA-4 and human VCAM. Reduced ligand affinities could make the binding of human effector cells to porcine endothelial cells more susceptible to disruption of any one of multiple weak interactions. These observations suggest the potential for significant differences in the therapeutic efficacy achieved by inhibiting particular receptor–ligand interactions with xenografts as compared to allografts.

Although the ch3F4 HuG4 and ch2A2HuG4 blocked binding of Jurkat and YT to activated PAEC, both antibodies failed to block binding of the monocytic U-937 cells to activated PAEC (Fig. 5). The levels of activated $\beta 1$ were comparable on Jurkat and U937 cells indicating an increased level of functional VLA-4 was not the mechanism behind the lack of inhibition of U-937 binding. Additionally, flow cytometry demonstrated the direct binding of the chimeric HuG4 antibodies to U-937 cells (Fig. 7). This binding was not dependent on the antigen specificity of the variable region, suggesting the interaction occurred through the Fc portion of the antibody. Finally, F(ab′)2 prepared from 3F4 HuG4 were able to block the adhesion of U-937 cells to activated PAEC by greater than 80% (Fig. 8). Together these results were consistent with a model in which the chimeric anti-pVCAM HuG4 mAbs crosslinked U-937 cells to activated PAEC by simultaneously binding FcRs and cell surface VCAM present on the PAEC. This is comparable to the situation encountered with the anti-CD3 murine monoclonal antibody (mAb) OKT3, which when first administered to patients produces crosslinking of T cells to FcR bearing cells, resulting in the activation of both cell types (Ceuppens *et al.*, 1985; Kan *et al.*, 1986) with resultant toxicity to the patient.

The interaction of antibodies with Fcγ receptors has been found to be dependent on the sequence of the hinge linker region of the CH2 domain (Duncan *et al.*, 1988; Alegre *et al.*, 1992; Burton and Woof, 1992) as well as an additional bend located between two β strands within the CH2 domain (Canfield and Morrison, 1991). In the case of a humanized OKT3 antibody (human IgG4 constant regions), binding to Fcγ receptors has been eliminated by the introduction of amino acid mutations in the hinge linker (Alegre *et al.*, 1992, 1994). In either case the resulting human IgG4 constant region contains amino acid differences which could be immunogenic. Here, we have created chimeric antibodies (designated HuG2/G4) containing the CH1 domain and hinge linker region of

Samsung Bioepis Exhibit No. 1002
Page 7676

human IgG2 followed by the CH2 and CH3 domains of the human IgG4 constant region. Due to the similarities between these two isotypes at the amino acid level, the resultant antibody contained no novel peptides less than 30 amino acids in length likely to be presented by MHC class II molecules and invoke an immune response. The chimeric HuG2/G4 antibodies no longer bound to U-937 cells. Moreover, the ch2A2 HuG2/G4 and ch3F4 HuG2/G4 antibodies blocked the binding of Jurkat and YT cells to activated PAEC as potently as the chimeric HuG4 antibodies, demonstrating the alteration of the Fc region did not reduce the antibody avidities. Moreover, the chimeric anti-pVCAM HuG2/G4 recombinant antibodies nearly completely blocked the binding of U-937 cells to activated PAEC and to purified pVCAM. Finally, as predicted from the low affinity of IgG2 and IgG4 for human C1q, our preliminary results indicate that the HuG4 and HuG2/G4 recombinant antibodies show little or no C1q binding (data not shown).

In summary, we have developed humanized mAbs specific for porcine VCAM which potently block the adhesion of multiple human inflammatory cell types to porcine endothelial cells. The lack of binding of these antibodies to human VCAM leaves intact the interaction of human leukocytes with human endothelial cells. The adhesion-blocking properties of these antibodies will allow delineation of the role of VLA-4/VCAM interactions in cellular mediated rejection of porcine-to-primate xenografts. Additionally, the HuG2/G4 antibody design should prove useful in humanization of other antibodies intended for human use where elimination of FcR binding and C activation may be desirable.

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Samsung Bioepis Exhibit No. 1002
Page 7677

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Samsung Bioepis Exhibit No. 1002
Page 7678

INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Q5C

Current *Step 4* version

dated 30 November 1995

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

Samsung Bioepis Exhibit No. 1002
Page 7679

Q5C
Document History

First Codification	History	Date	New Codification **November 2005**
Q5C	Approval by the Steering Committee under *Step 2* and release for public consultation.	29 March 1995	Q5C

Current *Step 4* version

Q5C	Approval by the Steering Committee under *Step 4* and recommendation for adoption to the three ICH regulatory bodies.	30 November 1995	Q5C

Samsung Bioepis Exhibit No. 1002
Page 7680

QUALITY OF BIOTECHNOLOGICAL PRODUCTS: STABILITY TESTING OF BIOTECHNOLOGICAL/BIOLOGICAL PRODUCTS

Annex to the Tripartite ICH Guideline for the Stability Testing of New Drug Substances and Products

ICH Harmonised Tripartite Guideline

Having reached *Step 4* of the ICH Process at the ICH Steering Committee meeting on 30 November 1995, this guideline is recommended for adoption to the three regulatory parties to ICH

1. PREAMBLE

The guidance stated in the ICH harmonised tripartite guideline "Stability Testing of New Drug Substances and Products" (27 October 1993) applies in general to biotechnological/biological products. However, biotechnological/biological products do have distinguishing characteristics to which consideration should be given in any well-defined testing program designed to confirm their stability during the intended storage period. For such products, in which the active components are typically proteins and/or polypeptides, maintenance of molecular conformation and, hence of biological activity, is dependent on noncovalent as well as covalent forces. The products are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. In order to ensure maintenance of biological activity and to avoid degradation, stringent conditions for their storage are usually necessary.

The evaluation of stability may necessitate complex analytical methodologies. Assays for biological activity, where applicable, should be part of the pivotal stability studies. Appropriate physicochemical, biochemical and immunochemical methods for the analysis of the molecular entity and the quantitative detection of degradation products should also be part of the stability program whenever purity and molecular characteristics of the product permit use of these methodologies.

With the above concerns in mind, the applicant should develop the proper supporting stability data for a biotechnological/biological product and consider many external conditions which can affect the product's potency, purity and quality. Primary data to support a requested storage period for either drug substance or drug product should be based on long-term, real-time, real-condition stability studies. Thus, the development of a proper long-term stability program becomes critical to the successful development of a commercial product. The purpose of this document is to give guidance to applicants regarding the type of stability studies that should be provided in support of marketing applications. It is understood that during the review and evaluation process, continuing updates of initial stability data may occur.

2. SCOPE OF THE ANNEX

The guidance stated in this annex applies to well-characterised proteins and polypeptides, their derivatives and products of which they are components, and which are isolated from tissues, body fluids, cell cultures, or produced using rDNA technology. Thus, the document covers the generation and submission of stability data for products such as cytokines (interferons, interleukins, colony-stimulating factors, tumour necrosis factors), erythropoietins, plasminogen activators, blood

Samsung Bioepis Exhibit No. 1002
Page 7681

plasma factors, growth hormones and growth factors, insulins, monoclonal antibodies, and vaccines consisting of well-characterised proteins or polypeptides. In addition, the guidance outlined in the following sections may apply to other types of products, such as conventional vaccines, after consultation with the appropriate regulatory authorities. The document does not cover antibiotics, allergenic extracts, heparins, vitamins, whole blood, or cellular blood components.

3. TERMINOLOGY

For the basic terms used in this annex the reader is referred to the "Glossary" in the ICH harmonised tripartite guideline "Stability Testing of New Drug Substances and Products" (27 October 1993). However, since manufacturers of biotechnological/biological products sometimes use traditional terminology, traditional terms are specified in parentheses to assist the reader. A supplemental glossary is also included that explains certain terms used in the production of biotechnological/biological products.

4. SELECTION OF BATCHES

4.1. Drug Substance (Bulk Material)

Where bulk material is to be stored after manufacture but prior to formulation and final manufacturing, stability data should be provided on at least 3 batches for which manufacture and storage are representative of the manufacturing scale of production. A minimum of 6 months stability data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug substances with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Data from pilot-plant scale batches of drug substance produced at a reduced scale of fermentation and purification may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long-term stability program after approval.

The quality of the batches of drug substance placed into the stability program should be representative of the quality of the material used in preclinical and clinical studies and of the quality of the material to be made at manufacturing scale. In addition, the drug substance (bulk material) made at pilot-plant scale should be produced by a process and stored under conditions representative of that used for the manufacturing scale. The drug substance entered into the stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size may be acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of container/closure system that is intended to be used during manufacture.

4.2. Intermediates

During manufacture of biotechnological/biological products, the quality and control of certain intermediates may be critical to the production of the final product. In general, the manufacturer should identify intermediates and generate in-house data and process limits that assure their stability within the bounds of the developed process. While the use of pilot-plant scale data is permissible, the manufacturer should establish the suitability of such data using the manufacturing scale process.

Samsung Bioepis Exhibit No. 1002
Page 7682

4.3. Drug Product (Final Container Product)

Stability information should be provided on at least 3 batches of final container product representative of that which will be used at manufacturing scale. Where possible, batches of final container product included in stability testing should be derived from different batches of bulk material. A minimum of 6 months data at the time of submission should be submitted in cases where storage periods greater than 6 months are requested. For drug products with storage periods of less than 6 months, the minimum amount of stability data in the initial submission should be determined on a case-by-case basis. Product expiration dating will be based upon the actual data submitted in support of the application. Since dating is based upon the real-time/real-temperature data submitted for review, continuing updates of initial stability data should occur during the review and evaluation process. The quality of the final container product placed on stability studies should be representative of the quality of the material used in the preclinical and clinical studies. Data from pilot-plant scale batches of drug product may be provided at the time the dossier is submitted to the regulatory agencies with a commitment to place the first 3 manufacturing scale batches into the long term stability program after approval. Where pilot-plant scale batches were submitted to establish the dating for a product and, in the event that product produced at manufacturing scale does not meet those long-term stability specifications throughout the dating period or is not representative of the material used in preclinical and clinical studies, the applicant should notify the appropriate regulatory authorities to determine a suitable course of action.

4.4. Sample Selection

Where one product is distributed in batches differing in fill volume (e.g., 1 millilitre (ml), 2 ml, or 10 ml), unitage (e.g., 10 units, 20 units, or 50 units), or mass (e.g., 1 milligram (mg), 2 mg, or 5 mg) samples to be entered into the stability program may be selected on the basis of a matrix system and/or by bracketing.

Matrixing, i.e., the statistical design of a stability study in which different fractions of samples are tested at different sampling points, should only be applied when appropriate documentation is provided that confirms that the stability of the samples tested represents the stability of all samples. The differences in the samples for the same drug product should be identified as, for example, covering different batches, different strengths, different sizes of the same closure and possibly, in some cases, different container/closure systems. Matrixing should not be applied to samples with differences that may affect stability, such as different strengths and different containers/closures, where it cannot be confirmed that the products respond similarly under storage conditions.

Where the same strength and exact container/closure system is used for 3 or more fill contents, the manufacturer may elect to place only the smallest and largest container size into the stability program, i.e., bracketing. The design of a protocol that incorporates bracketing assumes that the stability of the intermediate condition samples are represented by those at the extremes. In certain cases, data may be needed to demonstrate that all samples are properly represented by data collected for the extremes.

Samsung Bioepis Exhibit No. 1002
Page 7683

5. STABILITY-INDICATING PROFILE

On the whole, there is no single stability-indicating assay or parameter that profiles the stability characteristics of a biotechnological/biological product. Consequently, the manufacturer should propose a stability-indicating profile that provides assurance that changes in the identity, purity and potency of the product will be detected.

At the time of submission, applicants should have validated the methods that comprise the stability-indicating profile and the data should be available for review. The determination of which tests should be included will be product-specific. The items emphasised in the following subsections are not intended to be all-inclusive, but represent product characteristics that should typically be documented to adequately demonstrate product stability.

5.1. Protocol

The dossier accompanying the application for marketing authorisation should include a detailed protocol for the assessment of the stability of both drug substance and drug product in support of the proposed storage conditions and expiration dating periods. The protocol should include all necessary information which demonstrates the stability of the biotechnological/biological product throughout the proposed expiration dating period including, for example, well-defined specifications and test intervals. The statistical methods that should be used are described in the tripartite guideline on stability.

5.2. Potency

When the intended use of a product is linked to a definable and measurable biological activity, testing for potency should be part of the stability studies. For the purpose of stability testing of the products described in this guideline, potency is the specific ability or capacity of a product to achieve its intended effect. It is based on the measurement of some attribute of the product and is determined by a suitable quantitative method. In general, potencies of biotechnological/biological products tested by different laboratories can be compared in a meaningful way only if expressed in relation to that of an appropriate reference material. For that purpose, a reference material calibrated directly or indirectly against the corresponding national or international reference material should be included in the assay.

Potency studies should be performed at appropriate intervals as defined in the stability protocol and the results should be reported in units of biological activity calibrated, whenever possible, against nationally or internationally recognised standard. Where no national or international reference standards exist, the assay results may be reported in in-house derived units using a characterised reference material.

In some biotechnological/biological products, potency is dependent upon the conjugation of the active ingredient(s) to a second moiety or binding to an adjuvant. Dissociation of the active ingredient(s) from the carrier used in conjugates or adjuvants should be examined in real-time/real-temperature studies (including conditions encountered during shipment). The assessment of the stability of such products may be difficult since, in some cases, *in vitro* tests for biological activity and physicochemical characterisation are impractical or provide inaccurate results. Appropriate strategies (e.g., testing the product prior to conjugation/binding, assessing the release of the active compound from the second moiety, *in vivo* assays) or the use of an appropriate surrogate test should be considered to overcome the inadequacies of *in vitro* testing.

Samsung Bioepis Exhibit No. 1002
Page 7684

5.3. Purity and Molecular Characterisation

For the purpose of stability testing of the products described in this guideline, purity is a relative term. Due to the effect of glycosylation, deamidation, or other heterogeneities, the absolute purity of a biotechnological/biological product is extremely difficult to determine. Thus, the purity of a biotechnological/biological product should be typically assessed by more than one method and the purity value derived is method-dependent. For the purpose of stability testing, tests for purity should focus on methods for determination of degradation products.

The degree of purity, as well as individual and total amounts of degradation products of the biotechnological/biological product entered into the stability studies, should be reported and documented whenever possible. Limits of acceptable degradation should be derived from the analytical profiles of batches of the drug substance and drug product used in the preclinical and clinical studies.

The use of relevant physicochemical, biochemical and immunochemical analytical methodologies should permit a comprehensive characterisation of the drug substance and/or drug product (e.g., molecular size, charge, hydrophobicity) and the accurate detection of degradation changes that may result from deamidation, oxidation, sulfoxidation, aggregation or fragmentation during storage. As examples, methods that may contribute to this include electrophoresis (SDS-PAGE, immunoelectrophoresis, Western blot, isoelectrofocusing), high-resolution chromatography (e.g., reversed-phase chromatography, gel filtration, ion exchange, affinity chromatography), and peptide mapping.

Wherever significant qualitative or quantitative changes indicative of degradation product formation are detected during long-term, accelerated and/or stress stability studies, consideration should be given to potential hazards and to the need for characterisation and quantification of degradation products within the long-term stability program. Acceptable limits should be proposed and justified, taking into account the levels observed in material used in preclinical and clinical studies.

For substances that cannot be properly characterised or products for which an exact analysis of the purity cannot be determined through routine analytical methods, the applicant should propose and justify alternative testing procedures.

5.4. Other Product Characteristics

The following product characteristics, though not specifically relating to biotechnological/biological products, should be monitored and reported for the drug product in its final container:

> Visual appearance of the product (colour and opacity for solutions/suspensions; colour, texture and dissolution time for powders), visible particulates in solutions or after the reconstitution of powders or lyophilised cakes, pH, and moisture level of powders and lyophilised products.

Sterility testing or alternatives (e.g., container/closure integrity testing) should be performed at a minimum initially and at the end of the proposed shelf-life.

Additives (e.g., stabilisers, preservatives) or excipients may degrade during the dating period of the drug product. If there is any indication during preliminary stability studies that reaction or degradation of such materials adversely affect the quality of the drug product, these items may need to be monitored during the stability program.

The container/closure has the potential to adversely affect the product and should be carefully evaluated (see below).

Samsung Bioepis Exhibit No. 1002
Page 7685

6. STORAGE CONDITIONS

6.1. Temperature

Since most finished biotechnological/biological products need precisely defined storage temperatures, the storage conditions for the real-time/real-temperature stability studies may be confined to the proposed storage temperature.

6.2. Humidity

Biotechnological/biological products are generally distributed in containers protecting them against humidity. Therefore, where it can be demonstrated that the proposed containers (and conditions of storage) afford sufficient protection against high and low humidity, stability tests at different relative humidities can usually be omitted. Where humidity-protecting containers are not used, appropriate stability data should be provided.

6.3. Accelerated and Stress Conditions

As previously noted, the expiration dating should be based on real-time/real-temperature data. However, it is strongly suggested that studies be conducted on the drug substance and drug product under accelerated and stress conditions. Studies under accelerated conditions may provide useful support data for establishing the expiration date, provide product stability information for future product development (e.g., preliminary assessment of proposed manufacturing changes such as change in formulation, scale-up), assist in validation of analytical methods for the stability program, or generate information which may help elucidate the degradation profile of the drug substance or drug product. Studies under stress conditions may be useful in determining whether accidental exposures to conditions other than those proposed (e.g., during transportation) are deleterious to the product and also for evaluating which specific test parameters may be the best indicators of product stability. Studies of the exposure of the drug substance or drug product to extreme conditions may help to reveal patterns of degradation; if so, such changes should be monitored under proposed storage conditions. While the tripartite guideline on stability describes the conditions of the accelerated and stress study, the applicant should note that those conditions may not be appropriate for biotechnological/biological products. Conditions should be carefully selected on a case-by-case basis.

6.4. Light

Applicants should consult the appropriate regulatory authorities on a case-by-case basis to determine guidance for testing.

6.5. Container/Closure

Changes in the quality of the product may occur due to the interactions between the formulated biotechnological/biological product and container/closure. Where the lack of interactions cannot be excluded in liquid products (other than sealed ampoules), stability studies should include samples maintained in the inverted or horizontal position (i.e., in contact with the closure), as well as in the upright position, to determine the effects of the closure on product quality. Data should be supplied for all different container/closure combinations that will be marketed.

In addition to the standard data necessary for a conventional single-use vial, the applicant should demonstrate that the closure used with a multiple-dose vial is capable of withstanding the conditions of repeated insertions and withdrawals so that the product retains its full potency, purity, and quality for the maximum period

Samsung Bioepis Exhibit No. 1002
Page 7686

specified in the instructions-for-use on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

6.6. Stability after Reconstitution of Freeze-Dried Product

The stability of freeze-dried products after their reconstitution should be demonstrated for the conditions and the maximum storage period specified on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

7. TESTING FREQUENCY

The shelf-lives of biotechnological/biological products may vary from days to several years. Thus, it is difficult to draft uniform guidelines regarding the stability study duration and testing frequency that would be applicable to all types of biotechnological/biological products. With only a few exceptions, however, the shelf-lives for existing products and potential future products will be within the range of 0.5 to 5 years. Therefore, the guidance is based upon expected shelf-lives in that range. This takes into account the fact that degradation of biotechnological/biological products may not be governed by the same factors during different intervals of a long storage period.

When shelf-lives of 1 year or less are proposed, the real-time stability studies should be conducted monthly for the first 3 months and at 3 month intervals thereafter.

For products with proposed shelf-lives of greater than 1 year, the studies should be conducted every 3 months during the first year of storage, every 6 months during the second year, and annually thereafter.

While the testing intervals listed above may be appropriate in the pre-approval or pre-licence stage, reduced testing may be appropriate after approval or licensure where data are available that demonstrate adequate stability. Where data exist that indicate the stability of a product is not compromised, the applicant is encouraged to submit a protocol which supports elimination of specific test intervals (e.g., 9 month testing) for post-approval/post-licensure, long-term studies.

8. SPECIFICATIONS

Although biotechnological/biological products may be subject to significant losses of activity, physicochemical changes, or degradation during storage, international and national regulations have provided little guidance with respect to distinct release and end of shelf-life specifications. Recommendations for maximum acceptable losses of activity, limits for physicochemical changes, or degradation during the proposed shelf-life have not been developed for individual types or groups of biotechnological/biological products but are considered on a case-by-case basis. Each product should retain its specifications within established limits for safety, purity, and potency throughout its proposed shelf-life. These specifications and limits should be derived from all available information using the appropriate statistical methods. The use of different specifications for release and expiration should be supported by sufficient data to demonstrate that clinical performance is not affected as discussed in the tripartite guideline on stability.

9. LABELLING

For most biotechnological/biological drug substances and drug products, precisely defined storage temperatures are recommended. Specific recommendations should be stated, particularly for drug substances and drug products that cannot tolerate

Samsung Bioepis Exhibit No. 1002
Page 7687

freezing. These conditions, and where appropriate, recommendations for protection against light and/or humidity, should appear on containers, packages, and/or package inserts. Such labelling should be in accordance with relevant national/regional requirements.

10. GLOSSARY

Conjugated Product

A conjugated product is made up of an active ingredient (for example, peptide, carbohydrate) bound covalently or noncovalently to a carrier (for example, protein, peptide, inorganic mineral) with the objective of improving the efficacy or stability of the product.

Degradation Product

A molecule resulting from a change in the drug substance (bulk material) brought about over time. For the purpose of stability testing of the products described in this guideline, such changes could occur as a result of processing or storage (e.g., by deamidation, oxidation, aggregation, proteolysis). For biotechnological/biological products some degradation products may be active.

Impurity

Any component of the drug substance (bulk material) or drug product (final container product) which is not the chemical entity defined as the drug substance, an excipient, or other additives to the drug product.

Intermediate

For biotechnological/biological products, a material produced during a manufacturing process which is not the drug substance or the drug product but whose manufacture is critical to the successful production of the drug substance or the drug product. Generally, an intermediate will be quantifiable and specifications will be established to determine the successful completion of the manufacturing step prior to continuation of the manufacturing process. This includes material which may undergo further molecular modification or be held for an extended period of time prior to further processing.

Manufacturing Scale Production

Manufacture at the scale typically encountered in a facility intended for product production for marketing.

Pilot-Plant Scale

The production of the drug substance or drug product by a procedure fully representative of and simulating that to be applied at manufacturing scale. The methods of cell expansion, harvest, and product purification should be identical except for the scale of production.

Samsung Bioepis Exhibit No. 1002
Page 7688

J A • K E M P

21 November 2018

Our Ref: C411599EP

SUBMISSION UNDER R116

Patent No:	2359834
Application No:	11001632.6
Patentee:	Alexion Pharmaceuticals, Inc.
Opponents:	(1) Amgen Inc. (2) Hoffmann Eitle

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CONTENTS

1 REQUESTS 1

2 DOCUMENTS 1

3 DECLARATION OF DR KLIBANOV AND INVENTIVE STEP 2

4 INSUFFICIENCY 4

5 AUXILIARY REQUESTS 5

6 SUMMARY 5

Samsung Bioepis Exhibit No. 1002
Page 7690

1 REQUESTS

1.1 The Patent Proprietor requests that the Opposition Division maintains the Patent as granted.

1.2 If the Opposition Division is not able to grant the Main Request, even after discussion at Oral Proceedings, then the Patent Proprietor requests that the patent is maintained on the basis of one of Auxiliary Requests 1 or 2 enclosed herewith. Also enclosed, to assist the Opposition Division, are tracked copies of Auxiliary Requests 1 and 2 showing the amendments which have been made compared with the Main Request.

1.3 It is further requested that, should any minor alterations be required to these Auxiliary Requests, an opportunity be provided to make such amendments at the Oral Proceedings.

1.4 We shall speak in English at the Oral Proceedings. We shall require interpretation into English should any other party speak in a language other than English.

1.5 We shall be accompanied at the hearing by Todd Spalding and Tara Rahemba of Alexion Pharmaceuticals, Inc and Dr Dino Miano, also of Alexion Pharmaceuticals, Inc and author of declaration D33. I request permission for Dr Miano to speak at the oral proceedings, should this be appropriate. Dr Miano's qualifications are set out in D33. Dr Miano may speak in relation to the commercial product, Soliris. Dr Miano may also discuss the subject matter contained in his declaration, in particular the stability data presented therein, as well as the content of Dr Treuheit's declaration D23.

2 DOCUMENTS

2.1 The following additional documents are cited and enclosed herewith:

D39 Declaration of Dr Alexander Klibanov.

D40 Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8.

D41 Alberts *et al*, Molecular Biology of the Cell, Third Edition, 1994, page 585.

2.2 We also enclose a consolidated list of all the documents cited by both Opponents and the Patent Proprietor, including the additional documents mentioned above.

Samsung Bioepis Exhibit No. 1002
Page 7691

3 Declaration of Dr Klibanov and Inventive Step

3.1 Dr Klibanov is an expert in the formulation of therapeutic proteins, including antibodies. In his declaration, Dr Klibanov provides his expertise on the issues which the skilled person in this field would have faced around 2007, the filing date of the present patent. Dr Klibanov particularly comments on the complexities and challenges which the skilled person would have faced in 2007, when developing a therapeutic antibody formulation.

3.2 In the Summons, the Opposition Division summarise the arguments of the Patent Proprietor in the submission of 22 March 2018 and comment briefly on the Proprietor's position that formulating antibodies was a complex area. It is the Proprietor's position that the difficulties in antibody formulation in 2007 cannot be overestimated and it is considered important that the significance of these issues is fully appreciated. Accordingly, Dr Klibanov, who is an expert in this field and was working in this area in 2007, has been asked to provide his expert opinion.

3.3 The relevance of Dr Klibanov's declaration to the Patent Proprietor's position on inventive step will be explained further below with reference to the problem and solution approach:

3.4 The Opposition Division set out their view that D1 can be taken as the closest prior art document. The difference between the claimed subject matter and that of D1 is at least the following:

- The formulation is a single use 300mg dosage form;
- The concentration is 10 mg/ml;
- The volume is 30ml;
- The solution is preservative free.

3.5 As explained in detail in section 5.3 of the 22 March 2018 submission, the technical effect of the claimed composition is extremely good long term stability, such that the antibody maintains its integrity and biological activity in the composition. Further, the 10 mg/ml concentration provides this enhanced stability in a low-volume liquid formulation which is easy for the clinician to store and use.

3.6 Accordingly, the objective technical problem to be solved over D1 is to provide a composition of eculizumab, an antibody having a unique, engineered structure, which provides the ideal and necessary balance of properties for use in the treatment of PNH, including enhanced maintenance of biological integrity following prolonged storage, whilst also providing a low-volume liquid formulation.

Samsung Bioepis Exhibit No. 1002
Page 7692

3.7 The solution to this problem is the 300 mg single-use dosage form of eculizumab, comprising 30 ml of a 10 mg/ml preservative free solution.

3.8 The skilled person could not have predicted, at the filing date of the patent, that the 10 mg/ml composition would provide the required balance of low volume formulation combined with high stability. Indeed, as Dr Klibanov explains in his declaration at paragraph 4, achieving long term stability was a major challenge at the time:

"One of the major challenges in developing a successful pharmaceutical formulation of a therapeutic antibody (and of any therapeutic protein) around 2007 was, and still is today, being able to create a composition that is stable enough for sufficiently long periods of time such that the antibody does not undergo deleterious physical or chemical degradation reactions or form aggregates prior to administration."

3.9 Dr Klibanov comments that a skilled person faced with the task of formulating a new therapeutic protein would not have expected to be able to develop a liquid composition at all, due to these potential instabilities (see paragraph 6, D39). Dr Klibanov goes on to discuss the difficulties of balancing the various causes of protein instability, concluding in paragraph 10:

"Given the complexity and the sheer number of the various modes of protein instability and the diverse mechanisms underlying each of them, there was no universal approach to successfully formulating an antibody in 2007 (nor is there one now). My both academic and industrial experience at the time was that the field of antibody formulation was difficult, case-specific, and uncertain. For these reasons, a person skilled in the art aiming to develop a liquid formulation of a new antibody with a sufficient storage stability at a high concentration would not have been able to predict which, if any, such formulation would meet the necessary requirements."

3.10 Dr Klibanov's view therefore confirms the unpredictability of the field. The Opponents suggest that the composition of eculizumab described in claim 1 would have been obvious, based on disclosures relating to formulations of different antibodies, in particular D20 and D21. Dr Klibanov's experience, however, demonstrates that this is not the case: as he confirms, in fact, each antibody needs to be considered individually. This requires a uniquely-designed, detailed research program to determine the ideal composition for a given antibody. A person skilled in the art would not have had any confidence that applying a formulation found to be successful for a different antibody would also be successful with eculizumab.

3.11 In particular, in paragraphs 8 and 9 of his declaration, Dr Klibanov explains that each of D20 and D21 referred to by the Opponents highlight the uniqueness of every antibody and the unpredictable nature of formulating a stable antibody composition. D20 teaches that *"each antibody seems to have a unique personality related to its requirements for stability"* (see

Samsung Bioepis Exhibit No. 1002
Page 7693

paragraph 9 of D39), and D21 stresses *"that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the difference in their sequence"* (see paragraph 9 of D39).

3.12 Further, eculizumab itself would have presented particular difficulties to the skilled person in developing a suitable formulation. As set out in paragraph 3.6 above, the objective technical problem relates to providing a suitable formulation for eculizumab, which has a unique, engineered structure. Dr Klibanov explains at paragraphs 7 to 9 of D39 that even small structural differences between antibodies can lead to significant differences in behaviour in liquid compositions. For instance, at paragraph 7, Dr Klibanov refers to a 2002 text book:

"*The book explains that 'the exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation' and that '[e]ven for closely related proteins, the relative stability and major pathways for degradation might be quite different.' (Ex. 2 at 185-186.) In the 2007 time-frame, researchers like myself understood that therapeutic antibody (or protein in general) formulations, especially liquid ones, are* ***not*** *amenable to a 'one size fits all' approach."*

3.13 The sensitivity of protein stability to primary sequence, which Dr Klibanov here refers to, would have been particularly relevant to eculizumab, given its uniquely-engineered structure.

3.14 Dr Klibanov's declaration therefore demonstrates that the skilled person in the art in 2007, faced with the issue of developing a pharmaceutical composition for eculizumab which provides the right balance of a low volume formulation with high stability, would not have been able to predict whether a liquid composition would be possible at all, let alone be able to predict, with a reasonable expectation of success, that a 10 mg/ml formulation in particular would provide the required balance of properties. For these reasons, the Proprietor maintains that the claimed subject matter is in accordance with Article 56 EPC.

4 Insufficiency

4.1 The skilled person would have had no difficulty in carrying out the invention as claimed, which is to provide a single-use dosage form of a sterile and preservative free solution comprising 30ml of a 10mg eculizumab/ml solution and to use it to treat PNH patients. This is because the full heavy and light chain sequences of eculizumab are disclosed in SEQ ID NOs: 2 and 4 in the patent, enabling the skilled person to produce eculizumab.

4.2 Although SEQ ID NO: 4 in the patent is the *immature* light chain sequence of eculizumab and thus contains the signal peptide, the signal peptide would be cleaved off during antibody production in the cell, and this would have been known to the skilled person as part of his common general

Samsung Bioepis Exhibit No. 1002
Page 7694

knowledge at the effective filing date (see, for example, D41, an extract from the 1994 textbook, Alberts *et al*). Therefore, the skilled person would have been able to make (the mature form of) eculizumab, and hence a single-use dosage form of a sterile and preservative free solution comprising 30ml of a 10mg eculizumab/ml solution, without an undue burden in 2007.

5 Auxiliary Requests

5.1 Auxiliary Request 1 specifies that administration is by intravenous infusion. Basis for this amendment can be found on page 22 of the application as filed. For instance, page 22 lines 18 to 20 indicate that intravenous administration is the most preferable route of administration, and page 22, line 32 also specifies intravenous infusion.

5.2 Auxiliary Request 2 specifies that administration is via IV infusion and that the composition is diluted to a final concentration of 5 mg/ml prior to administration. Basis for this amendment can be found at page 22, lines 27 to 29 of the application as filed.

5.3 Each of these Auxiliary Requests is filed in order to address the arguments raised by the Opponents in relation to inventive step.

6 Summary

6.1 For the reasons discussed above and in our previous response, the Proprietor requests that the patent is maintained as granted and the oppositions are dismissed.

Signed

AMANDA SIMONS
J A KEMP
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PAMELA TUXWORTH
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ASSOCIATION NO. 811

Samsung Bioepis Exhibit No. 1002
Page 7695

AUXILIARY REQUEST 1

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein administration is by intravenous infusion.

Samsung Bioepis Exhibit No. 1002
Page 7696

AUXILIARY REQUEST 2

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein the composition is administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration.

Samsung Bioepis Exhibit No. 1002
Page 7697

AUXILIARY REQUEST 1

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein administration is by intravenous infusion.

Samsung Bioepis Exhibit No. 1002
Page 7698

AUXILIARY REQUEST 2

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein the composition is administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration.

Samsung Bioepis Exhibit No. 1002
Page 7699

Consolidated List of Documents cited in opposition to EP2359834 B1				
Document Name	Consolidated Number	Patentee	Opponent 1 (Amgen)	Opponent 2 (Hoffman Eitle)
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Samsung Bioepis Exhibit No. 1002
Page 7700

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Commercial monoclonal antibodies 2006 (I.V. administration)	D32			Annex II
Declaration of Dr Miano	D33	D33		
Rouet *et al.*, (2014) *FEBS Letters.* 588: 269-277	D34	D34		
Summary of antibodies listed in D20/D21 Tables	D35	D35		
Graphical representation of D20 Table 2	D36	D36		
Summary of antibody products approved by EMA since filing date	D37	D37		
Extracts from Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991)	D38	D38		
Declaration of Dr Alexander Klibanov	D39	D39		
Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8.	D40	D40		
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Samsung Bioepis Exhibit No. 1002
Page 7701

MOLECULAR BIOLOGY OF
THE CELL
THIRD EDITION

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson



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Samsung Bioepis Exhibit No. 1002
Page 7702

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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

tein translocator. There is now strong evidence for a protein translocator. Normally, the pore in the translocator is plugged with the growing polypeptide chain that is in transit across the membrane; when the nascent chains are experimentally released from the ribosomes with the drug puromycin, however, the pores can be detected by the ion currents that flow through them. Although the pore is large enough to allow the passage of an unfolded polypeptide chain, it closes when the ribosome is removed from the membrane (Figure 12–42). Thus the pore seems to be a dynamic structure, opening when a ribosome with a growing polypeptide chain attaches to the membrane and closing when the ribosome detaches after the synthesis of the protein is completed.

The ER Signal Peptide Is Removed from Most Soluble Proteins After Translocation [31]

We have seen that in chloroplasts and mitochondria the signal peptides are cleaved from the precursor proteins once they have crossed the membrane. Similarly, amino-terminal ER signal peptides are removed by a signal peptidase on the luminal side of the ER membrane. The peptide by itself, however, is not sufficient to signal cleavage by the peptidase; this requires an adjacent cleavage site that is specifically recognized by the peptidase. We shall see below that ER signal peptides that are contained within the polypeptide chain rather than at the amino terminus do not have these recognition sites and are never cleaved; instead, they can serve to retain transmembrane proteins in the lipid bilayer after the translocation process has been completed.

The amino-terminal ER signal peptide of a soluble protein itself has two signaling functions: in addition to directing the protein to the ER membrane, it is thought to serve as a **start-transfer signal,** which remains bound to the translocation apparatus while the rest of the protein is threaded continuously through the membrane as a large loop. Once the carboxyl terminus of the protein has passed through the membrane, the signal peptide is released from the translocator pore, cleaved off by the signal peptidase, and rapidly degraded to amino acids by other proteases in the ER while the protein is released into the ER lumen (Figure 12–43).

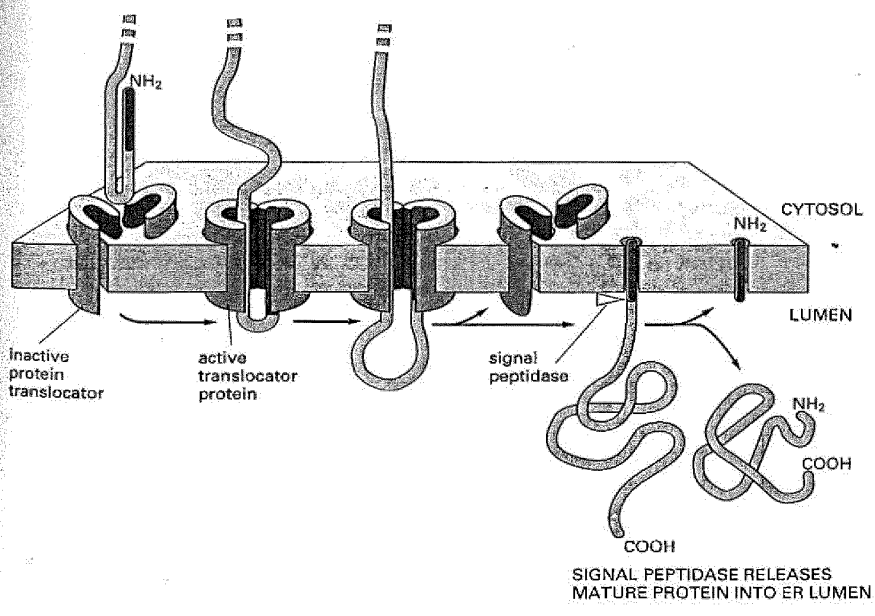


Figure 12–43 **The translocation of a soluble protein across the ER membrane.** In this hypothetical model the protein translocator in the membrane is postulated to exist in two alternative states—active or inactive. On binding an ER signal peptide (which acts as a start-transfer signal), the translocator adopts an active state and begins to transfer the polypeptide chain across the lipid bilayer as a loop. In this state it forms an aqueous pore across the membrane that can be detected electrophysiologically if the polypeptide chain is released (see Figure 12–42). After the protein has been completely translocated, the translocator reverts to an inactive conformation, which can no longer conduct ions across the membrane but is open to the lipid bilayer, allowing the hydrophobic signal peptide to diffuse out into the bilayer, where it is rapidly degraded. In this and the following two figures the ribosomes have been omitted for clarity.

Samsung Bioepis Exhibit No. 1002
Page 7704

IN THE MATTER OF
European Patent No. EP 2 359 834

DECLARATION OF PROFESSOR ALEXANDER M. KLIBANOV

I, Dr. Alexander M. Klibanov, hereby declare as follows:

1. I am a Professor of Chemistry and Bioengineering at the Massachusetts Institute of Technology ("M.I.T."), where I have been teaching and conducting research for more than 39 years. I currently hold the Novartis Endowed Chair Professorship. I obtained an M.S. degree in Chemistry in 1971 and a Ph.D. in Chemical Enzymology in 1974, both from Moscow University in Russia.

2. Throughout my career as a practicing chemist, I have extensively researched, published, taught, and lectured in many areas of biological, medicinal, protein, and pharmaceutical formulation chemistry. For my professional accomplishments, I have received numerous honors. For example, I was elected to the U.S. National Academy of Sciences and the U.S. National Academy of Engineering; I have also received various awards and prizes from the American Chemical Society, as well as other national and international organizations. I serve on the Editorial Boards of over a dozen scientific journals, including the "Journal of Biological Chemistry and Molecular Pharmacology" and the "International Journal of Drug Design, Delivery and Safety." I have published some 315 scientific papers in various chemical disciplines, including protein stability and stabilization, as well as drug formulation and delivery. I have also founded six pharmaceutical companies and have been on the scientific advisory boards and/or boards of directors of those companies, as well as of many others. A number of these advisory and directorship activities have involved the formulation, stability, stabilization, administration, and pharmacological evaluation of protein-based therapeutic agents, including antibodies. A copy of my curriculum vitae is attached as Exhibit 1.

3. I have been asked by Alexion to provide information regarding the field of protein, including antibody, pharmaceutical formulations as of 2007 and what understanding and expectations a person skilled in that field would have had concerning their stability at that time.

4. One of the major challenges in developing a successful pharmaceutical formulation of a therapeutic antibody (and of any therapeutic protein) around 2007 was, and still is today, being able to create a composition that is stable enough for sufficiently long periods of time such that the antibody does not undergo deleterious physical or chemical degradation reactions or form aggregates prior to administration. If the antibody is not adequately stable in the composition, serious problems can arise, such as drastically diminished efficacy and adverse immune responses in the patient. Moreover, when developing an antibody for commercial therapeutic use, it is often critical to be able to formulate the antibody at a rather high concentration (thus allowing a low-volume dosage).

5. Based on my professional experience, in the 2007 time-frame (or even today) there was no assurance that a skilled person, when provided with a new therapeutic protein, including an antibody, would have been able to develop a suitably stable pharmaceutical composition to make it commercially viable. My experience in this regard is confirmed by relevant scientific publications around that time period, as discussed below.

6. For example, a 2002 book entitled "Rational Design of Stable Protein Formulations. Theory and Practice" (the "2002 book;" Ex. 2) describes the uncertainty that existed in the protein formulation field regarding the ability to develop a suitably stable liquid pharmaceutical composition for a new protein. The authors of the 2002 book state that "protein drugs may not be stable enough to be handled as a liquid formulation" and concede that "with many proteins, it is not possible – especially considering the time constraints with product development – to develop sufficiently stable aqueous formulations." (Ex. 2 at 10 and 109.) The authors suggested that there was no reasonable expectation at the time of being able to develop such a composition, especially a liquid one, *e.g.*, stating that "[i]t can be *assumed* that most proteins will not exhibit sufficient stability in aqueous solution to allow a liquid formulation to be developed." (Ex. 2 at 188.) Consequently, most pharmaceutical proteins, such as antibodies, commercially available even today are in a lyophilized form, which requires their reconstitution prior to administration, thereby making it far less convenient and more expensive than aqueous solutions.

7. As the 2002 book recognized and was understood both then and now, numerous factors influence the stability of an antibody (or any protein), thus making the development of a suitable pharmaceutical composition in each new specific case a unique challenge. The book explains that "the exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation" and that "[e]ven for closely related proteins, the relative stability and major pathways for degradation might be quite different." (Ex. 2 at 185-186.) In the 2007 time-frame, researchers like myself understood that therapeutic antibody (or protein in general) formulations, especially liquid ones, are ***not*** amenable to a "one size fits all" approach. Therefore, when given a new antibody and tasked with creating its suitable pharmaceutical composition, a person skilled in the art would have had little guidance in that endeavor. Indeed, the 2002 book recognized that developing such a stable composition "is not a simple task" and that "for most proteins, maintaining physical and chemical stabilities in aqueous solution for an extended period of time is extremely difficult" (Ex. 2 at 10-11 and 184).

8. A review article entitled "Formulation and delivery issues for monoclonal antibody therapeutics" published in 2006 in a peer-reviewed international journal had recognized the uniqueness of each therapeutic antibody's challenge when developing a suitably robust formulation: "A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most[,] if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for

stability." (D21 at 690). For this reason, proteins typically have to be evaluated individually and stabilized on an empirical basis, which inevitably leads to unpredictability.

9. Another, 2007 review paper entitled "Antibody structure, instability, and formulation" points out that "antibodies, like other proteins, may generate a variety of degradants during production, processing, and storage both in liquid and solid states. Their tendency to generate such degradants depends very much on individual sequence, pI, hydrophobicity, and carbohydrate content." (D20 at 14). The review noted numerous variables affecting each of the many causes of antibody instability; because they were, in turn, influenced by a number of intertwining factors, as well as by the structure of the protein molecule itself, a person skilled in the art in 2007 would have recognized that predicting in advance whether or how a new antibody could be stabilized under relevant conditions at a particular concentration in a pharmaceutical composition was not possible: "It should be stressed that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the difference in their sequence." (D20 at 14).

10. Given the complexity and the sheer number of the various modes of protein instability and the diverse mechanisms underlying each of them, there was no universal approach to successfully formulating an antibody in 2007 (nor is there one now). My both academic and industrial experience at the time was that the field of antibody formulation was difficult, case-specific, and uncertain. For these reasons, a person skilled in the art aiming to develop a liquid formulation of a new antibody with a sufficient storage stability at a high concentration would ***not*** have been able to predict which, if any, such formulation would meet the necessary requirements.

I do hereby declare that the facts stated above are true to the best of my knowledge.



Dr. Alexander M. Klibanov

November 16, 2018

Date

Samsung Bioepis Exhibit No. 1002
Page 7707

Exhibit 1: Dr Klibanov Curriculum Vitae

Exhibit 2: Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8 (D40).

Samsung Bioepis Exhibit No. 1002
Page 7708

Curriculum vitae

ALEXANDER M. KLIBANOV

Date and Place of Birth: July 15, 1949, in Moscow (Russia)

Nationality: Naturalized U.S. Citizen (1983)

Education:

1974	Ph.D. in Chemical Enzymology, Moscow University
1971	M.S. in Chemistry, Moscow University

Honors:

2015	Tau Beta Pi's Leonardo Da Vinci Lecturer, MIT
2007-11 and 2014-	Novartis Chair Endowed Professorship, MIT
2012-13	Roger and Georges Firmenich Endowed Professorship, MIT
2011	MIT Biological Engineering Senior Class Faculty Award
2006	Distinguished GRUM Lecturer in Drug Discovery & Development, University of Montreal (Canada)
2004	UNAM Distinguished Lecturer, National University of Mexico (Mexico City)
2001	Walter Enz Lecturer in Pharmaceutical Chemistry, University of Kansas
2001	Elected a Corresponding Fellow of the Royal Society of Edinburgh (Scotland's National Academy of Science and Letters)
2000	Merck Distinguished Lecturer, Rutgers University
2000	Top 20 *Biotechnology & Bioengineering* Papers of the Last Forty Years
1998	Robert Lutz Lecturer, University of Virginia
1996	Perkin-Elmer Distinguished Lecturer, University of Pittsburgh
1995	Elected to the National Academy of Sciences of the U.S.A.
1995	Nathan O. Kaplan Memorial Lecturer in Biological Chemistry, University of California at San Diego
1994	R.W. Johnson PRI Lecturer, Pharmaceutical Research Institute
1994	Warren McCabe Lecturer, North Carolina State University
1993	Elected to the National Academy of Engineering of the U.S.A.
1993	Arthur C. Cope Scholar Award of the American Chemical Society
1993	Biotechnology Divisional Lectureship Award of the Institute of Food Technologists
1992	Charles Sabat Lecturer, Rutgers University
1992	Elected a Founding Fellow of the American Institute for Medical and Biological Engineering
1992	Louis C. Jordy Research Scholar Lecturer, Drew University
1991	International Enzyme Engineering Award
1991	Marvin J. Johnson Award of the American Chemical Society
1990	Monsanto Lecturer, Ohio State University
1990	NRC Distinguished Lecturer, Academia Sinica (Taiwan)
1989	Ipatieff Prize of the American Chemical Society
1989	Backer Lecturer, Groningen University (Holland)
1988	Dow Lecturer, University of Ottawa (Canada)
1987	Distinguished Scholar Lecturer, Hope College
1986	Leo Friend Award of the American Chemical Society
1984	Who's Who in Frontier Science and Technology

Momenta Pharm. v. BMS, IPR215-01537
1/41 BMS EXHIBIT 2016

2263

Samsung Bioepis Exhibit No. 1002
Page 7709

1984	Sohio Lecturer, Case Western Reserve University
1982	American Men and Women of Science
1981-1983	Henry L. Doherty Career Development Professorship, MIT
1975	U.S.S.R. Ministry of Higher Education Prize

Professional Experience:

2007-11 and 2014-	Novartis Chair Endowed Professor of Chemistry and Bioengineering, MIT
2012-2013	Roger and Georges Firmenich Professor of Natural Products Chemistry Department of Chemistry, MIT
2000-present	Professor of Bioengineering Department of Biological Engineering, MIT
1988-present	Professor of Chemistry Department of Chemistry, MIT
1987-1988	Professor of Applied Biochemistry Department of Applied Biological Sciences, MIT
1983-1987	Associate Professor of Applied Biochemistry Department of Applied Biological Sciences (formerly Department of Nutrition and Food Science), MIT
1979-1983	Assistant Professor of Applied Biochemistry Department of Nutrition and Food Science, MIT
1977-1979	Postdoctoral Associate, Department of Chemistry University of California at San Diego
1974-1977	Research Chemist Department of Chemistry, Moscow University

Current Journal Editorial/Advisory Boards:

"Biocatalysis and Biotransformation", "Applied Biochemistry and Biotechnology", "Central European Journal of Chemistry", "Biotechnology Progress", "Biotechnology & Bioengineering", "Microbial Biotechnology", "Open Journal of Pharmacology", "Nanocarriers", "Journal of Antivirals and Antiretrovirals", "Open Access Academic Books in Chemistry", "Journal of Biological Chemistry and Molecular Pharmacology", "Recent Patents in Biotechnology", "Current Pharmaceutical Biotechnology"

Professional Societies:

U.S. National Academy of Sciences, U.S. National Academy of Engineering, American Chemical Society, Society for Applied Microbiology

Current Research Interests:

Enzyme chemistry and biotechnology
Medicinal and formulation chemistry
Protein drug delivery
Antimicrobial polymers
Enzymes as stereoselective catalysts in organic syntheses
Stabilization and formulation of macromolecular pharmaceuticals

Momenta Pharm. v. BMS, IPR215-01537
2/41 BMS EXHIBIT 2016

2264

Samsung Bioepis Exhibit No. 1002
Page 7710

Publications:

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Momenta Pharm. v. BMS, IPR215-01537
3/41 BMS EXHIBIT 2016

2265

Samsung Bioepis Exhibit No. 1002
Page 7711

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Momenta Pharm. v. BMS, IPR215-01537
4/41 BMS EXHIBIT 2016

2266

Samsung Bioepis Exhibit No. 1002
Page 7712

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Momenta Pharm. v. BMS, IPR215-01537
5/41 BMS EXHIBIT 2016

2267

Samsung Bioepis Exhibit No. 1002
Page 7713

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6/41 BMS EXHIBIT 2016

2268

Samsung Bioepis Exhibit No. 1002
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7/41 BMS EXHIBIT 2016

2269

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8/41 BMS EXHIBIT 2016

2270

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9/41 BMS EXHIBIT 2016

2271

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10/41 BMS EXHIBIT 2016

2272

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11/41 BMS EXHIBIT 2016

2273

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12/41 BMS EXHIBIT 2016

2274

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13/41 BMS EXHIBIT 2016

2275

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14/41 BMS EXHIBIT 2016

2276

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2277

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2278

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2279

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2280

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19/41 BMS EXHIBIT 2016

2281

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2282

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2283

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2284

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305. Gerrard, S.E., Larson, A.M., Klibanov, A.M., Slater, N.K.H., Hanson, C.V., Abrams, B.F., Morris, M.K. 2013. Reducing infectivity of HIV upon exposure to surfaces coated with *N*,*N*-dodecyl,methyl-polyethylenimine. Biotechnol. Bioeng. 110: 2058-2062.

306. Larson, A.M., Chen, J., Klibanov, A.M. 2013. Conjugation to polymeric chains of influenza drugs targeting M2 ion channels partially restores inhibition of drug-resistant mutants. J. Pharm. Sci. 102: 2450-2459.

307. Weight, A.K., Belser, J.A., Tumpey, T.M., Chen, J., Klibanov, A.M. 2013. Zanamivir conjugated to poly-L-glutamine is much more active against influenza viruses in mice and ferrets than the drug itself. Pharm. Res. 31: 466-474.

308. Tandon, A., Sharma, A., Rodier, J.T., Klibanov, A.M., Rieger, F.G., Mohan, R.R. 2013. BMP7 gene transfer via gold nanoparticles into stroma inhibits corneal fibrosis *in vivo*. PLOS ONE 8(6): e66434, 1-9.

309. Liu, H., Kim, Y., Mello, K., Lovaasen, J., Shah, A., Rice, N., Yim, J.H., Pappas, D., Klibanov, A.M. 2014. Aerosol-assisted plasma deposition of hydrophobic polycations makes surfaces highly antimicrobial. Appl. Biochem. Biotechnol. 172: 1254-1264.

310. Zhu, A., Liu, H.K., Long, F., Su, E., Klibanov, A.M. 2015. Biocidal action of electric current applied to composite membranes containing carbon nanotubes. Appl. Biochem. Biotechnol. 175: 666-676.

Momenta Pharm. v. BMS, IPR215-01537
23/41 BMS EXHIBIT 2016

2285

Samsung Bioepis Exhibit No. 1002
Page 7731

311. Liu, K., Elkin, I., Chen, J., Klibanov, A.M. 2015. Why do some immobilized *N*-alkylated polyethylenimines far surpass others in inactivating influenza viruses? Biomacromolecules 16: 351-356.

312. Su, E., Klibanov, A.M. 2015. Low-transition-temperature mixtures (LTTMs) for dissolving proteins and for drug formulation. Appl. Biochem. Biotechnol. 177: 753-758.

313. Elkin, I., Weight, A.K., Klibanov, A.M. 2015. Markedly lowering the viscosity of aqueous solutions of DNA by additives. International J. Pharmaceutics 494: 66-72.

314. Kumar, A., Klibanov, A.M. 2015. Bulky organic salts drastically lower viscosities of concentrated suspensions of bacterial cells. Biotechnol. Bioeng., submitted.

Patents:

1. Klibanov, A.M., Langer, R.S. Methods of decreasing the hydrophobicity of fibroblast and other interferons. U.S. Patent No. 4,414,147; November 8, 1983.

2. Klibanov, A.M., Kirchner, G. Enzymatic production of optical isomers of 2-halopropionic acids. U.S. Patent No. 4,601,987; July 22, 1986.

3. Klibanov, A.M. Removal of combined organic substances from aqueous solutions. U.S. Patent No. 4,623,465; November 18, 1986.

4. Klibanov, A.M. Enzymatic resolution of racemic mixtures of hydroxy compounds. U.S. Patent No. 4,659,671; 1987.

5. Klibanov, A.M., Dordick, J.S. Enzymatic temperature change indicator. U.S. Patent No. 4,826,762; May 2, 1989.

6. Dorval, B.L., Denham, L., Klibanov, A.M. Radial flow assay, delivering membrane, test kit, and methods. U.S. Patent No. 5,547,833; August 20, 1996.

7. Dorval, B.L., Denham, L., Klibanov, A.M. Detection reagent, particle, and immunoassay method. U.S. Patent No. 5,561,045; October 1, 1996.

8. Dorval, B.L., Chow, M., Klibanov, A.M. Stabilized vaccine compositions. U.S. Patent No. 5,618,539; April 8, 1997. European Patent No. 0 487 632; March 4, 1998.

9. Klibanov, A.M., Lewis, K., Ferrante, A.A., Coyle, C.L., Zylstra, G., Logan, M.S.P., Grossman, M.J. Solvent-resistant microorganisms. U.S. Patent No. 5,807,735; September 15, 1998.

10. Gresser, J.D., Trantolo, D.J., Langer, R., Klibanov, A.M., Wise, D.L. Material for buffered resorbable internal fixation devices and method for making the same. U.S. Patent No. 5,817,328; October 6, 1998.

11. Hekal, I.M., Langer, R.S., Klibanov, A.M., Mathiowitz, E. Monolithic polymer composition having a water absorption material. U.S. Patent No. 6,174,952; January 16, 2001.

Momenta Pharm. v. BMS, IPR215-01537
24/41 BMS EXHIBIT 2016

2286

Samsung Bioepis Exhibit No. 1002
Page 7732

12. Gresser, J.D., Trantolo, D.J., Langer, R., Lewandrowski, K.-U., Klibanov, A.M., Wise, D.L. Resorbable interbody spinal fusion devices. U.S. Patent No. 6,241,771; June 5, 2001.

13. Trantolo, D.J., Langer, R., Klibanov, A.M., Wise, D.L. Buffered resorbable internal fixation devices and methods for making material therefor. U.S. Patent No. 6,419,945; July 16, 2002.

14. Gresser, J.D., Trantolo, D.J., Langer, R., Lewandrowski, K.-U., Klibanov, A.M., Wise, D.L. Method of making biodegradable interbody spinal fusion devices. U.S. Patent No. 6,548,002; April 15, 2003.

15. Klibanov, A.M. Nonaqueous solutions and suspensions of macromolecules for pulmonary delivery. U.S. Patent No. 6,660,715; December 9, 2003.

16. Tiller, J.C., Lewis, K., Liao, C.-J., Klibanov, A.M. Antimicrobial polymeric surfaces. U.S. Patent No. 7,151,139; December 19, 2006.

17. Hirsh, J.C., Fleming, A.B., Rariy, R.V., Klibanov, A.M. Abuse-deterrent pharmaceutical formulations. U.S. Patent No. 8,449,909; May 28, 2013.

18. Rariy, R.V., Fleming, A.B., Hirsh, J.C., Klibanov, A.M. Abuse-deterrent pharmaceutical compositions of opioids and other drugs. U.S. Patent No. 8,557,291; October 15, 2013.

19. Hirsh, J.C., Klibanov, A.M., Swager, T.M., Buchwald, S.L., Lo, W.Y., Fleming, Rariy, R.V. Abuse-deterrent pharmaceutical compositions of opioids and other drugs. U.S. Patent No. 9,044,398; June 2, 2015.

20. Schaer, T.P., Stewart, S., Klibanov, A.M. Antibacterial coatings that inhibit biofilm formation on implants. U.S. Patent No. 9,089,407; July 28, 2015.

21. Schaer, P.T., Hsu, B.B., Stewart, S., Klibanov, A.M. Hydrophobic polycationic coatings that inhibit biofilm formation on orthopedic implants and support healing during infection, pending.

22. Haldar, J., Chen, J., Klibanov, A.M. Non-leaching virucidal coatings, pending.

23. Haldar, J., An, D., Álvarez de Cienfuegos, L., Chen, J., Klibanov, A.M. Polymeric coatings that inactivate viruses and bacteria. U.S. Patent Appl. Publ. No. 2010/0136072; June 3, 2010.

24. Pedersen, M., Stanley, M., Gouin, S., Klibanov, A.M, Hsu, B.B. Antimicrobial quat coating on fabrics. PCT Intl. Appl. WO 2012/06561, 2012.

25. Haldar, J., Alvarez de Cienfuegos, L., Klibanov, A.M., Chen, J. Bi-functional polymer-attached inhibitors of influenza virus. PCT Intl. Appl. WO 2009/032605, 2012.

Invited Lectures:

1. Gordon Conference on Immobilized Enzymes, Complexes, and Cells (Plymouth, NH). August 1978.

2. 5th International Conference on Enzyme Engineering (Henniker, NH). August 1978.

Momenta Pharm. v. BMS, IPR215-01537
25/41 BMS EXHIBIT 2016

2287

Samsung Bioepis Exhibit No. 1002
Page 7733

3. Miles Laboratories (Elkhart, IN). February 1980.
4. Gordon Conference on Immobilized Species (Henniker, NH). August 1980.
5. Novo Laboratories (Wilton, CT). October 1980.
6. Corning Glass Works Co. (Corning, NY). October 1980.
7. Stauffer Chemical Co. (Dobbs Ferry, NY). December 1980.
8. DuPont Experimental Station (Wilmington, DE). January 1981.
9. Dow Chemical Co. (Wayland, MA). April 1981.
10. Cetus Corp. (Berkeley, CA). April 1981.
11. Exxon Research & Engineering Co. (Linden, NJ). April 1981.
12. Meeting of the American Solar Energy Society (Philadelphia, PA). May 1981.
13. 6th International Conference on Enzyme Engineering (Kashikojima, Japan). September 1981.
14. Genex Corp. (Rockville, MD). October 1981.
15. ILP Symposium for Senior Executives (London, England). November 1981.
16. California Institute of Technology (Pasadena, CA). December 1981.
17. Symposium of the American Society for Microbiology (Atlanta, GA). March 1982.
18. Kodak Research Center (Rochester, NY). March 1982.
19. Repligen Corp. (Cambridge, MA). April 1982.
20. Symposium on the Biological Basis of New Developments in Biotechnology (Minneapolis, MN). May 1982.
21. Meeting of the Biochemical Society (Oxford, England). July 1982.
22. Meeting of the American Chemical Society (Kansas City, MO). September 1982.
23. Rotenburger Symposium on Enzyme Technology (Kassel, Germany). September 1982.
24. Genetics Institute (Boston, MA). September 1982.
25. Meeting of the American Institute of Chemical Engineers (Los Angeles, CA). November 1982.
26. G.D. Searle Co. (Chicago, IL). October 1982.
27. Halcon International Research Center (Montvale, NJ). October 1982.

Momenta Pharm. v. BMS, IPR215-01537
26/41 BMS EXHIBIT 2016

2288

Samsung Bioepis Exhibit No. 1002
Page 7734

28. International Workshop on Immobilized Enzymes and Cells (Bangkok, Thailand). December 1982.

29. Amoco Research Center (Naperville, IL). January 1983.

30. University College London (London, England). May 1983.

31. International Conference on Biotechnology Biotech '83 (London, England). May 1983.

32. Tufts University Workshop on Immobilized Enzymes and Proteins (Medford, MA). June 1983.

33. Gordon Conference on Organic Reactions and Processes (New Hampton, NH). July 1983.

34. Symposium "Biocatalysis: New Discoveries and Uses", Meeting of the American Society for Industrial Microbiology (Sarasota, FL). August 1983.

35. Monsanto Co. (St. Louis, MO). September 1983.

36. Pasteur Biosciences '83 Conference (Paris, France). September 1983.

37. 7th International Conference on Enzyme Engineering (White Haven, PA). September 1983.

38. University of Iowa (Iowa City, IA). October 1983.

39. CPC Moffett Technical Center (Summitt-Argo, IL). December 1983.

40. General Foods Co. (Dobbs Ferry, NY). December 1983.

41. U.S. National Bureau of Standards (Gaithersburg, MD). April 1984.

42. Millipore Corp. (Bedford, MA). April 1984.

43. Technical University of Munich (Munich, F.R.G.). May 1984.

44. Danish Academy of Technical Sciences (Copenhagen, Denmark). May 1984.

45. Novo International Biotechnology Symposium (Copenhagen, Denmark). May 1984.

46. CRA Scientific Conference (St. Charles, IL). June 1984.

47. Gordon Conference on Immobilized Species in Biotechnology (Plymouth, NH). August 1984.

48. Meeting of the American Chemical Society (Philadelphia, PA). August 1984.

49. International Biotechnology Conference Biotech '84 (Washington, DC). September 1984.

50. Staley Manufacturing Co. (Decatur, IL). October 1984.

51. Case Western Reserve University (Cleveland, OH). October 1984.

Momenta Pharm. v. BMS, IPR215-01537
27/41 BMS EXHIBIT 2016

2289

Samsung Bioepis Exhibit No. 1002
Page 7735

52. IMC Corp. (Terre Haute, IN). October 1984.
53. Syracuse University (Syracuse, NY). November 1984.
54. National University of Mexico (Mexico City, Mexico). November 1984.
55. National Starch & Chemical Corp. (Bridgewater, NJ). November 1984.
56. Mead Corp. (Chillicothe, OH). November 1984.
57. Hebrew University of Jerusalem (Jerusalem, Israel). November 1984.
58. U.S. Army Chemical R&D Center (Aberdeen Proving Ground, MD). December 1984.
59. Allied Corp. (Morristown, NJ). December 1984.
60. Bristol Laboratories (Syracuse, NY). January 1985.
61. University of Rochester (Rochester, NY). January 1985.
62. G.D. Searle Co. (Chicago, IL). February 1985.
63. Wellcome Research Laboratories (Research Triangle Park, NC). February 1985.
64. UCLA Symposium on Protein Structure, Folding and Design (Keystone, CO). April 1985.
65. International Conference on Application of Biocatalysts in Organic Synthesis (Noordwijkerhout, Holland). April 1985.
66. Guido Donegani Research Institute (Novara, Italy). April 1985.
67. Hoffmann-LaRoche Co. (Basel, Switzerland). April 1985.
68. Pittsburgh-Cleveland Catalysis Society Meeting (Pittsburgh, PA). May 1985.
69. Ethyl Corp. (Baton Rouge, LA). May 1985.
70. Stony Brook Symposium on Protein Engineering (Stony Brook, NY). May 1985.
71. Amgen Corp. (Thousand Oaks, CA). June 1985.
72. Gordon Conference on Applied Microbiology (New London, NH). August 1985.
73. International Congress of Biochemistry (Amsterdam, Holland). August 1985.
74. IUPAC Congress (Manchester, England). September 1985.
75. International Enzyme Engineering Conference (Helsingor, Denmark). September 1985.
76. Biotechnica '85 (Hannover, West Germany). October 1985.

Momenta Pharm. v. BMS, IPR215-01537
28/41 BMS EXHIBIT 2016

2290

Samsung Bioepis Exhibit No. 1002
Page 7736

77. Biotec 85 (Dusseldorf, West Germany). October 1985.

78. Smith Kline & French Co. (Philadelphia, PA). November 1985.

79. Exxon Corporate Research (Anandale, NJ). December 1985.

80. Pennsylvania State University (University Park, PA). December 1985.

81. Georgetown University (Washington, DC). February 1986.

82. Columbia University (New York, NY). February 1986.

83. Upjohn Co. (Kalamazoo, MI). February 1986.

84. UNIDO Workshop on Biotechnology (Trieste, Italy). March 1986.

85. Enericherche (Monterotondo, Italy). March 1986.

86. EEC Biomolecular Engineering Meeting (Compiegne, France). April 1986.

87. ACS Conference on Chemical Aspects of Biotechnology (Gaithersburg, MD). May 1986.

88. Biotech '86 Europe International Conference (London, England). May 1986.

89. IUPAC International Symposium on Organic Chemistry in Technological Perspective (Jerusalem, Israel). June 1986.

90. University of Tel-Aviv (Ramat-Aviv, Israel). June 1986.

91. Annual Meeting of the Canadian Biochemical Society (Guelph, Canada). June 1986.

92. International Symposium on Biologically Engineered Polymers (Cambridge, England). July 1986.

93. TNO Institute of Applied Chemistry (Zeist, The Netherlands). August 1986.

94. IUPAC International Symposium on the Chemistry of Natural Products (The Hague, The Netherlands). August 1986.

95. American Chemical Society National Meeting (Anaheim, CA). September 1986.

96. Catalytica Annual Science and Technology Symposium (San Jose, CA). September 1986.

97. International Conference on Enzyme Engineering (Cambridge, England). September 1986.

98. Ohio State University (Columbus, OH). October 1986.

99. Michigan State University (Lansing, MI). October 1986.

100. Biotechnology Process Engineering Symposium (Cambridge, MA). October 1986.

Momenta Pharm. v. BMS, IPR215-01537
29/41 BMS EXHIBIT 2016

2291

Samsung Bioepis Exhibit No. 1002
Page 7737

101. University of Delaware (Newark, DE). November 1986.

102. American Red Cross Blood Research Institute (Bethesda, MD). November 1986.

103. International Symposium on Biocatalysis in Non-Aqueous Solvents (Wageningen, The Netherlands). December 1986.

104. University of Trondheim (Trondheim, Norway). December 1986.

105. Colgate-Palmolive Research Center (Piscataway, NJ). January 1987.

106. International Paper Co. (Tuxedo Park, NY). January 1987.

107. University of Massachusetts (Amherst, MA). February 1987.

108. American Society for Microbiology Biocatalysis Symposium (Atlanta, GA). March 1987.

109. Hope College (Holland, MI). March 1987.

110. Pfizer Corporate Research Center (Groton, CT). April 1987.

111. OBBS Symposium on Protein Engineering (Ottawa, Canada). April 1987.

112. University of Texas (Austin, TX). April 1987.

113. Florida Catalysis Conference (Palm Coast, FL). May 1987.

114. PPG Industries (Barberton, OH). May 1987.

115. Abbott Laboratories (North Chicago, IL). May 1987.

116. American Oil Chemists Society Symposium on Lipases (New Orleans, LA). May 1987.

117. Argonne National Laboratory (Argonne, IL). June 1987.

118. Canadian Chemical Conference (Quebec City, Canada). June 1987.

119. Squibb Co. (New Brunswick, NJ). June 1987.

120. FEBS Annual Meeting (Ljubljana, Yugoslavia). July 1987.

121. L'Oreal Research Institute (Paris, France). July 1987.

122. International Symposium on Biointeractions (Cambridge, England). July 1987.

123. Eli Lilly Co. (Indianapolis, IN). August 1987.

124. Upjohn Co. (Kalamazoo, MI). September 1987.

125. International Enzyme Engineering Conference (Santa Barbara, CA). October 1987.

Momenta Pharm. v. BMS, IPR215-01537
30/41 BMS EXHIBIT 2016

2292

Samsung Bioepis Exhibit No. 1002
Page 7738

126. Shell Development Co. (Houston, TX). October 1987.

127. DuPont Experimental Station (Wilmington, DE). October 1987.

128. University of Helsinki (Helsinki, Finland). November 1987.

129. International Conference on Bioreactors and Biotransformations (Gleneagles, Scotland). November 1987.

130. Sandoz Co. (Basel, Switzerland). November 1987.

131. University of Waterloo (Waterloo, Canada). December 1987.

132. Norwegian Biochemical Society Annual Meeting (Bejito, Norway). January 1988.

133. Rutgers University (New Brunswick, NJ). February 1988.

134. University of Ottawa (Ottawa, Canada). February 1988.

135. Boston Biomedical Research Institute (Boston, MA). March 1988.

136. Princeton University (Princeton, NJ). March 1988.

137. Royal Society of Chemistry Meeting (Kent, England). April 1988.

138. Stanford University (Stanford, CA). May 1988.

139. University of Graz (Graz, Austria). May 1988.

140. Belgian Organic Chemistry Symposium (Ghent, Belgium). May 1988.

141. Dow Chemical Co. (Midland, MI). May 1988.

142. Michigan State University (Lansing, MI). May 1988.

143. American Chemical Society Meeting (Toronto, Canada). June 1988.

144. Proctor & Gamble Co. (Cincinnati, OH). June 1988.

145. International Congress of Biochemistry (Prague, Czechoslovakia). July 1988.

146. International Biotechnology Symposium (Paris, France). July 1988.

147. Gordon Conference on Catalysis (Newport, RI). August 1988.

148. Conference on Biocatalytic Synthesis of Organic Compounds (Saratoga, NY). August 1988.

149. International Symposium on Thermodynamics Applied to Biological Systems (Santa Margherita, Italy). September 1988.

150. Cornell University (Ithaca, NY). September 1988.

Momenta Pharm. v. BMS, IPR215-01537
31/41 BMS EXHIBIT 2016

2293

Samsung Bioepis Exhibit No. 1002
Page 7739

151. Eastman Kodak Co. (Kingsman, TN). September 1988.

152. Symposium on Biotechnology Challenges in the Food Industry (Williamsburg, VA). October 1988.

153. University of California at Irvine (Irvine, CA). October 1988.

154. UCSF Symposium on Protein and Drug Design (San Francisco, CA). October 1988.

155. Boston University (Boston, MA). October 1988.

156. Wayne State University (Detroit, MI). November 1988.

157. Kyoto Symposium on Frontiers in Biocatalysis (Kyoto, Japan). November 1988.

158. Rohm & Haas Co. (Spring House, PA). January 1989.

159. Block Drug Co. (Jersey City, NJ). January 1989.

160. State University of New York (Albany, NY). March 1989.

161. Scripps Clinic and Research Foundation (La Jolla, CA). April 1989.

162. Texas A&M University (College Station, TX). May 1989.

163. University of Texas (Austin, TX). May 1989.

164. International Congress of Food Engineering (Cologne, F.R.G.). May 1989.

165. University of Groningen (Groningen, The Netherlands). June 1989.

166. Gordon Conference on Enzymes, Coenzymes, and Metabolic Pathways (Meridan, NH). July 1989.

167. American Peptide Symposium (La Jolla, CA). July 1989.

168. International Symposium on Prospects in Protein Engineering (Haren, The Netherlands). August 1989.

169. Connaught Laboratories (Toronto, Canada). September 1989.

170. University of Georgia (Athens, GA). October 1989.

171. Spanish National Congress of Biochemistry (Alicante, Spain). October 1989.

172. Symposium on Receptor- and Enzyme-Based Drug Design (Chapel Hill, NC). October 1989.

173. Wesleyan University (Middletown, CT). November 1989.

174. University of Wisconsin (Madison, WI). February 1990.

175. Merck Sharp & Dohme Research Laboratories (Rahway, NJ). February 1990.

Momenta Pharm. v. BMS, IPR215-01537
32/41 BMS EXHIBIT 2016

2294

Samsung Bioepis Exhibit No. 1002
Page 7740

176. International Symposium on Biochemical Engineering (Stuttgart, F.R.G.). March 1990.

177. University of Geneva (Geneva, Switzerland). March 1990.

178. University of Lausanne (Lausanne, Switzerland). March 1990.

179. University of Bern (Bern, Switzerland). March 1990.

180. University of Fribourg (Fribourg, Switzerland). March 1990.

181. International Conference on Protein Stability (Cambridge, England). March 1990.

182. AFRC Institute of Food Research (Reading, England). March 1990.

183. Lehigh University (Bethlehem, PA). April 1990.

184. Biotechnology Research Institute (Montreal, Canada). April 1990.

185. Ohio State University (Columbus, OH). April 1990.

186. Pharmaceutical Manufacturers Association Annual Meeting (St. Louis, MO). April 1990.

187. International Conference on Industrial Use of Enzymes (Chicago, IL). May 1990.

188. Korea Advanced Institute of Science and Technology (Seoul, Korea). June 1990.

189. Academia Sinica and National Taiwan University (Taipei, Taiwan). June 1990.

190. Gordon Conference on Biocatalysis (Plymouth, NH). June 1990.

191. IUPAC Conference on Organic Synthesis (Helsinki, Finland). July 1990.

192. International Conference on Solution Chemistry (Ottawa, Canada). August 1990.

193. Gordon Conference on Biomolecular Recognition (Plymouth, NH). August 1990.

194. University of Naples (Naples, Italy). September 1990.

195. International Conference on Water and Life (Crans-sur-Sierre, Switzerland). September 1990.

196. International Symposium on Thermodynamic Basis of Protein Structure and Function (Kansas City, MO). October 1990.

197. Johns Hopkins University (Baltimore, MD). October 1990.

198. Institute of Medicine Workshop on Microheterogeneity of Biological Macromolecules (Arlington, VA). November 1990.

199. U.S.-Japan Biotechnology Conference (Honolulu, HI). January 1991.

Momenta Pharm. v. BMS, IPR215-01537
33/41 BMS EXHIBIT 2016

2295

Samsung Bioepis Exhibit No. 1002
Page 7741

200. Ciba-Geigy Corp. (Summit, NJ). February 1991.

201. City University of New York (New York, NY). March 1991.

202. Rensselaer Polytechnic Institute (Troy, NY). March 1991.

203. Bristol-Myers Squibb Co. (Wallingford, CT). April 1991.

204. British Biochemical Society Meeting (Reading, England). April 1991.

205. University of California at Berkeley (Berkeley, CA). April 1991.

206. California Institute of Technology (Pasadena, CA). April 1991.

207. Merck, Sharp & Dohme Research Laboratories (West Point, PA). May 1991.

208. Bristol-Myers Squibb Research Institute (Princeton, NJ). May 1991.

209. International Conference on Biocatalysis (Orlando, FL). June 1991.

210. U.S. Army Research, Development and Engineering Center (Natick, MA). July 1991.

211. Gordon Conference on Natural Products (Plymouth, NH). July 1991.

212. Lederle-Praxis Biologicals Co. (Sanford, NC). July 1991.

213. International Biochemistry Congress (Jerusalem, Israel). August 1991.

214. American Chemical Society Meeting (New York, NY). August 1991.

215. International Enzyme Engineering Conference (Kona, HI). September 1991.

216. University of Lowell (Lowell, MA). October 1991.

217. Rutgers University (Piscataway, NJ). November 1991.

218. International Symposium on Enzymes in Organic Synthesis (New Delhi, India). January 1992.

219. International BioSymposium Nagoya '92 (Nagoya, Japan). January 1992.

220. International Symposium on Biomolecules in Organic Solvents (Taxco, Mexico). February 1992.

221. Rockefeller University (New York, NY). March 1992.

222. Symposium on Molecular Chirality of the Pharmaceutical Society of Japan (Tokyo, Japan). April 1992.

223. Conference on Enzymes in Organic Chemistry of the Dutch Chemical Society (Wageningen, The Netherlands). April 1992.

Momenta Pharm. v. BMS, IPR215-01537
34/41 BMS EXHIBIT 2016

2296

Samsung Bioepis Exhibit No. 1002
Page 7742

224. International Symposium on Bioprocessing of Coal (Clearwater Beach, FL). May 1992.

225. Exxon Workshop on New Leads in Coal Depolymerization (Montgomery, TX). May 1992.

226. Italian Symposium on Biochemical Biotechnology (Capri, Italy). June 1992.

227. Gordon Conference on Biocatalysis (Meriden, NH). July 1992.

228. FEBS Meeting (Dublin, Ireland). August 1992.

229. International Biotechnology Symposium (Washington, DC). August 1992.

230. International Symposium Bio Japan '92 (Yokohama, Japan). August 1992.

231. Texas College of Osteopathic Medicine (Fort Worth, TX). October 1992.

232. Rutgers University (Newark, NJ). October 1992.

233. Schering-Plough Co. (Bloomfield, NJ). November 1992.

234. International Symposium ISOPOW-V: *Properties of Water in Foods* (Peniscola, Spain). November 1992.

235. Carlsberg Laboratory (Copenhagen, Denmark). November 1992.

236. International Food Technology Conference (The Hague, The Netherlands). November 1992.

237. Singapore Institute of Standards and Industrial Research (Singapore). November 1992.

238. Drew University (Madison, NJ). December 1992.

239. Northeastern University (Boston, MA). January 1993.

240. Ciba Corning Diagnostics Corp. (Irvine, CA). January 1993.

241. University of Kansas (Lawrence, KS). January 1993.

242. 6th International Symposium on Recent Advances in Drug Delivery Systems (Salt Lake City, UT). February 1993.

243. Pennsylvania State University (University Park, PA). March 1993.

244. Northwestern University (Evanston, IL). March 1993.

245. International Symposium on Technologies for the Production of Enantiomerically Pure Chemicals (Amelia Island, FL). March 1993.

246. Gordon Conference on Bioanalytical Sensors (Ventura, CA). March 1993.

247. Pharmaceutical Manufacturers Association Analytical R&D/Pharmaceutical Development Subsections Joint Annual Meeting (Lake Buena Vista, FL). April 1993.

Momenta Pharm. v. BMS, IPR215-01537
35/41 BMS EXHIBIT 2016

2297

Samsung Bioepis Exhibit No. 1002
Page 7743

248. University of Massachusetts Symposium on Breakthrough Technologies: *Paradigms for a New Century* (Amherst, MA). May 1993.

249. Parke-Davis Corp. (Ann Arbor, MI). May 1993.

250. NutraSweet Co. (Mt. Prospect, IL). June 1993.

251. IGT/GRI Workshop on Novel Concepts for the Production of Regio- and Stereospecific Compounds (Chicago, IL). June 1993.

252. Northeast Regional Meeting of the American Chemical Society (Boston, MA). June 1993.

253. Institute of Food Technologists' Biotechnology Meeting (Chicago, IL). July 1993.

254. Annual Meeting of Department of Energy's Biological and Chemical Technology Research Program Principal Investigators (Tiburon, CA). July 1993.

255. Allied-Signal Corp. (Des Plains, IL). July 1993.

256. American Chemical Society Meeting (Chicago, IL). August 1993.

257. University of Illinois at Urbana-Champaign (Urbana, IL). September 1993.

258. International Enzyme Engineering Conference (Deauville, France). September 1993.

259. Queens College (Flushing, NY). November 1993.

260. Exxon Chemical Research Meeting (Galveston, TX). November 1993.

261. Hoffmann-LaRoche Co. (Nutley, NJ). December 1993.

262. U.S.-Japan Symposium on Drug Delivery (Maui, HI). December 1993.

263. North Dakota State University (Fargo, ND). February 1994.

264. Annual Meeting of the American Institute for Medical and Biological Engineering (Washington, DC). March 1994.

265. Rhone-Poulenc Rorer Symposium on Visions in Chemistry (Collegeville, PA). May 1994.

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Momenta Pharm. v. BMS, IPR215-01537
36/41 BMS EXHIBIT 2016

2298

Samsung Bioepis Exhibit No. 1002
Page 7744

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Momenta Pharm. v. BMS, IPR215-01537
37/41 BMS EXHIBIT 2016

2299

Samsung Bioepis Exhibit No. 1002
Page 7745

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Momenta Pharm. v. BMS, IPR215-01537
38/41 BMS EXHIBIT 2016

2300

Samsung Bioepis Exhibit No. 1002
Page 7746

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Momenta Pharm. v. BMS, IPR215-01537
39/41 BMS EXHIBIT 2016

2301

Samsung Bioepis Exhibit No. 1002
Page 7747

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Momenta Pharm. v. BMS, IPR215-01537
40/41 BMS EXHIBIT 2016

2302

Samsung Bioepis Exhibit No. 1002
Page 7748

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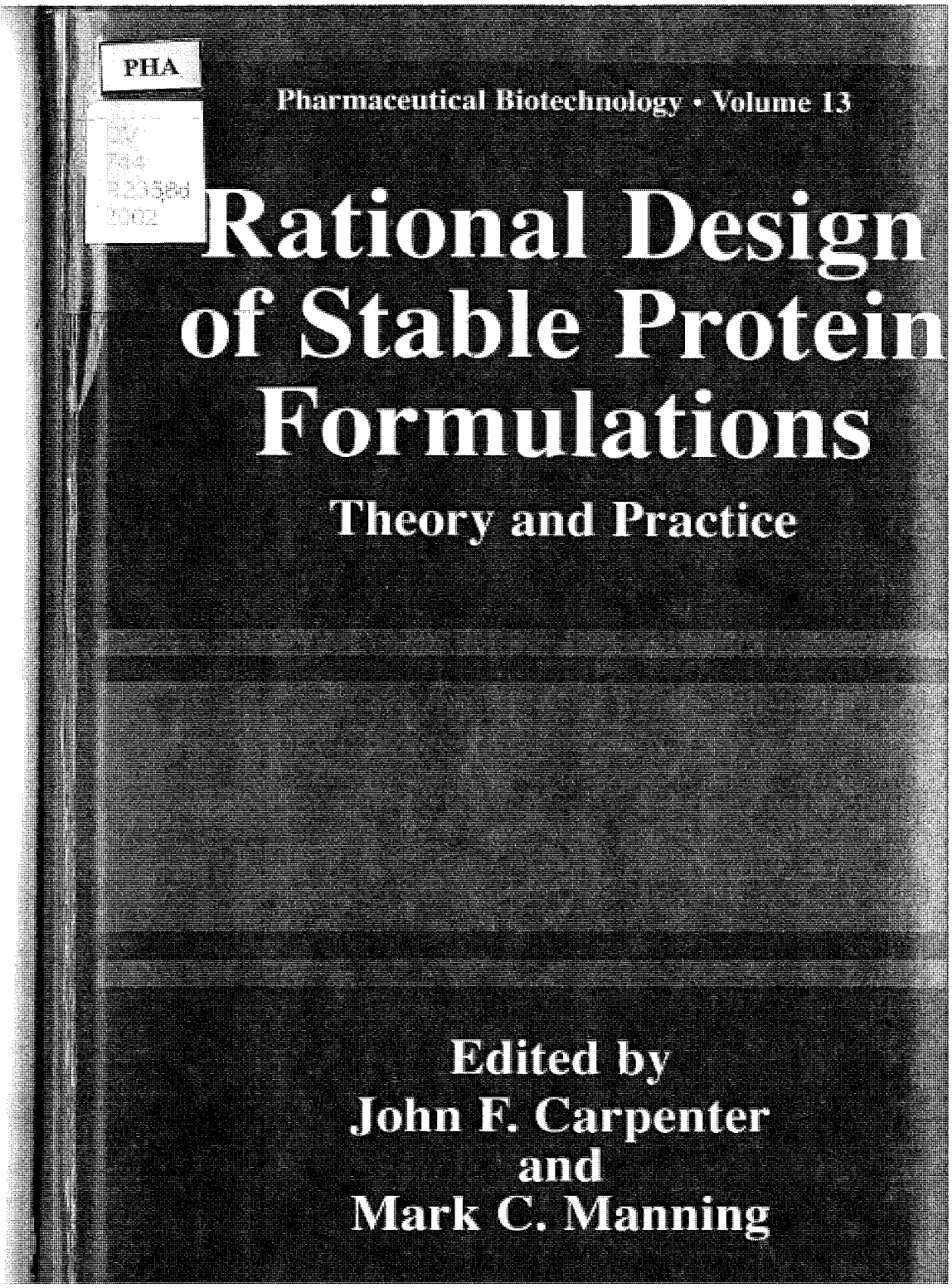
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Momenta Pharm. v. BMS, IPR215-01537
41/41 BMS EXHIBIT 2016

2303

Samsung Bioepis Exhibit No. 1002
Page 7749



Samsung Bioepis Exhibit No. 1002
Page 7750

Rational Design of Stable Protein Formulations

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Samsung Bioepis Exhibit No. 1002
Page 7753

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Theory and Practice

Edited by

John F. Carpenter

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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
Page 7755

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Samsung Bioepis Exhibit No. 1002
Page 7756

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Samsung Bioepis Exhibit No. 1002
Page 7757

Contents

Chapter 1

Practical Approaches to Protein Formulation Development
Byeong S. Chang and Susan Hershenson

Introduction 1
Preparation for Formulation Development 3
Resource Requirements for Formulation Development 3
Useful Information for Designing Formulations 4
Preformulation Development 4
Characterization of Protein Pharmaceuticals 5
Accelerated Stability Studies 5
Development of Analytical Methods 6
Evaluation of the Significance of Problems 7
Formulation Development 10
Formulation Options for Protein Pharmaceuticals 10
Typical Protein Stability Problems: Causes and Solutions 13
Optimization of Formulation Variables 13
Necessary Studies for Formulation Development 15
Strategies to Overcome Difficult Formulation Problems 17
Formulation in Commercial Product Development 18
Critical Formulation Decisions During Pharmaceutical Development 18
Formulation for Early Preclinical and Clinical Studies 19
Commercial Formulation 19
Regulatory Issues in Formulation Development 20

Samsung Bioepis Exhibit No. 1002
Page 7758

Appendix: List of Regulatory Documents 22
References 23

Chapter 2

Recombinant Production of Native Proteins from *Escherichia coli*
Tsutomu Arakawa, Tiansheng Li, and Linda O. Narhi

Introduction 27
Distribution of Expressed Proteins 28
Cell Washing and Lysis 32
Purification of Soluble, Folded Proteins 34
Purification and Refolding of Soluble, Misfolded Proteins 35
Purification and Refolding of Proteins from Inclusion Bodies 36
Washing and Solubilization of Inclusion Bodies 36
Purification of Expressed Proteins from Inclusion Bodies 36
Refolding Mechanism 38
Disulfide Bond Formation 41
Removal of Denaturant 41
Effects of Tag Sequences 44
Effects of Excipients 44
Response Surface Methodology 47
High Pressure Disaggregation and Refolding 48
Methods to Analyze Folded Structures 48
Bioactivity 49
Binding to Receptors 49
Dilsulfide Bond Analysis 50
Spectroscopy 50
Conformational Stability 51
Limited Proteolysis 51
References 51

Chapter 3

Physical Stabilization of Proteins in Aqueous Solution
Brent S. Kendrick, Tiansheng Li, and Byeong S. Chang

Introduction 61
Overview of Physical Stability 62

Samsung Bioepis Exhibit No. 1002
Page 7759

Thermodynamic Control of Protein Stability 62
Kinetic Control of Protein Stability 63
Interactions of Excipients with Proteins 65
Preferentially Excluded Cosolvents 66
Buffers/Salts 67
Specific Binding of Ligands 68
Protein Self-Stabilization 69
Physical Factors Affecting Protein Stability 70
Temperature 70
Freeze-Thawing 71
Agitation and Exposure to Denaturing Interfaces 71
Pressure 72
Conclusions 73
Appendix: Derivation of the Wyman Linkage Function and Application to the Timasheff Preferential Exclusion Mechanism 73
References 78

Chapter 4

Effects of Conformation on the Chemical Stability of Pharmaceutically Relevant Polypeptides
Jeffrey D. Meyer, Bert Ho, and Mark C. Manning

Introduction 85
Relationship Between Structure and Deamidation Rates 86
Primary Structure Effects 87
Secondary Structure Effects 89
Tertiary Structure Effects 91
Summary of Structure Effects on Deamidation 92
Role of Structure in Protein Oxidation 92
Types of Oxidation Processes 93
Effects of Oxidation of Surface and Buried Methionines on Protein Structure 95
Limiting Solvent Accessibility of Residues 96
Conformational Control of Oxidation in Aqueous Solution 97
Structural Control of Oxidation in Lyophilized Products 99
Summary of Structural Control of Oxidation 100
Summary 101
References 101

Samsung Bioepis Exhibit No. 1002
Page 7760

Chapter 5

Rational Design of Stable Lyophilized Protein Formulations: Theory and Practice
John F. Carpenter, Beyong S. Chang, William Garzon-Rodriguez, and Theodore W. Randolph

Introduction 109
Minimal Criteria for a Successful Lyophilized Formulation 111
Inhibition of Lyophilization-Induced Protein Unfolding 112
Storage at Temperatures Below Formulation Glass Transition Temperature 113
The Water Content is Relatively Low 114
A Strong, Elegant Cake Structure is Obtained 114
Steps Taken to Minimize Specific Routes of Protein Chemical Degradation 116
Rational Design of Stable Lyophiilized Formulations 117
Choice of Buffer 118
Specific Ligands/pH that Optimizes Thermodynamic Stability of Protein 119
Trehalose or Sucrose to Inhibit Protein Unfolding and Provide Glassy Matrix 120
Bulking Agent (e.g., Mannitol, Glycine or Hydroxyethyl Starch) 126
Nonionic Surfactant to Inhibit Aggregation. 127
Acknowledgments 127
References 127

Chapter 6

Spray-Drying of Proteins
Geoffrey Lee

Introduction: Why Spray-Dry a Protein? 135
Developments in the Last 10 Years 136
The Practice of Spray-Drying Proteins 139
Type of Equipment 139
Spray-Drying Conditions 140
Influence of Formulation 147
Pure Proteins 147
Formulated Systems 149
Use of Added Surface Active Substances 151

Samsung Bioepis Exhibit No. 1002
Page 7761

Concluding Remarks 156
References 156

Chapter 7

Surfactant-Protein Interactions
Theodore W. Randolph and LaToya S. Jones

Introduction 159
Proteins and Surfactants at Surfaces 161
Protein-Surfactant Interactions in Solution 166
Surfactant Effects on Protein Assembly State 167
Surfactant Effects on Proteins During Freezing, Freeze-Drying and Reconstitution 169
Enzymatic Degradation of Non-Ionic Surfactants 170
Recommendations for Protein Formulation 170
References 171

Chapter 8

High Throughput Formulation: Strategies for Rapid Development of Stable Protein Products
Rajiv Nayar and Mark C. Manning

Introduction 177
Overall Structure of the HTF Approach 179
Role of an Established Decision Tree for Formulation Design 181
Constraints on a Pharmaceutically Acceptable Protein Formulation 182
Proper Choice of Dosage Form 183
Preformulation Studies 185
Proper Choice of Excipients 186
Estimates of Resources Needed for Formulation Development 188
Use of Software and Databases to Assist in the HTF Process 189
Essential Analytical Methods 191
Stability Protocols 193
Unified Strategy for HTF 194
References 195

Index 199

Samsung Bioepis Exhibit No. 1002
Page 7762

1

Practical Approaches to Protein Formulation Development

Byeong S. Chang and Susan Hershenson

INTRODUCTION

As is the case with other pharmaceuticals, formulation development is one of the critical steps in developing a protein as a therapeutic product. Development of stable protein formulations may require even more resources and effort than conventional small molecule pharmaceuticals. Proteins typically have more stability issues as a result of their complexity and delicate structural stability. Fortunately, a great deal of research regarding protein stability has been conducted and this information is readily available in the literature (reviewed by Manning et al., 1989; Chen, 1992; Ahern and Manning, 1992a, 1992b; Arakawa et al., 1993; Cleland et al., 1993; Wang and Pearlman, 1993; Pearlman and Wang, 1996; Volkin and Middaugh, 1997). Ultimately, it would be ideal to be able to develop a pure pharmaceutical containing only the native protein. However, it is not practical to have only the native form of a protein in the formulation because the protein must be purified from a complex biological mixture containing a pool of other proteins which includes misfolded, denatured, and degraded forms of the same protein. Furthermore, a major challenge is to maintain the integrity of the purified protein during routine pharmaceutical processing, storage, handling, and delivery to the patient. One could envision achieving this goal by developing a formulation with

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Samsung Bioepis Exhibit No. 1002
Page 7763

perfect stability, i.e., no physical and chemical change in the protein. Because proteins are complex molecules composed of numerous reactive chemical groups and delicate three-dimensional structures, identifying a set of conditions to keep all components stable is virtually impossible. In general, commercial therapeutic protein formulations are developed under the assumption that some degree of physicochemical changes will occur during storage and handling.

Realizing that it is impossible to develop a perfectly stable formulation, especially while meeting an aggressive product development timeline, the main objective then becomes one of maintaining the appropriate safety and efficacy of the product. In order to achieve this objective, it is imperative to understand the broad spectrum of degradation pathways affecting proteins, and to have available equipment and expertise in an extensive repertoire of analytical methods. Formulation development focuses on determining the potential degradation pathways, assessing the significance of each and optimizing variables to minimize the degradation products that are clinically significant.

Regulatory guidelines also are critical elements for guiding formulation development. They provide information about how to conduct studies and obtain useful results for evaluating formulations. The results obtained allow formulation scientists to write an appropriate developmental pharmaceutics section in regulatory filings. The guidelines also help to evaluate the significance of some inevitable degradation products that are produced during manufacturing, shipping and storage. For example, if the degradation products have properties comparable to those of the desired product with respect to activity, efficacy, and safety, they can be classified as product-related substances. This classification is significantly different from considering the degradation product as an impurity when there is not sufficient supporting evidence to justify classification as a product-related substance (Appendix, Regulatory Document 1). Understanding of these practical issues of regulatory requirements is critical for formulation scientists during design, implementation, evaluation and reporting of their studies.

In addition to insight into the scientific and regulatory issues, developing commercial formulations requires a clear understanding of the potential market. For example, indication, patients, method of delivery, frequency of dosing, typical dose requirement, market distribution and other business-related information will provide directions for the design of a successful formulation. Also, it is important to consider the competitiveness of the formulation as compared to other products available in the market.

In this chapter, an overview of critical factors affecting the design of therapeutic protein formulations and a general guide to developing commercially viable dosage forms for protein pharmaceuticals will be discussed. Since the majority of practical issues are not covered very well in the scientific literature, this chapter also includes information from regulatory guidance documents (see Appendix), labels from marketed products and routine industrial experience.

Samsung Bioepis Exhibit No. 1002
Page 7764

PREPARATION FOR FORMULATION DEVELOPMENT

Resource Requirements for Formulation Development

A list of resources that should be available before starting formulation development is summarized in Table 1. Some resources may not be as critical as others, depending on the nature of problems encountered, but it is important to have sufficient resources to discover major formulation issues as early as possible in the product development process. Without the appropriate equipment and personnel, the development of an acceptable formulation can be greatly hampered, potentially to such a degree that the product is never brought to market. Of course, not all of the resources need to be at the company developing the therapeutic protein, but if outside contractors are employed it is essential that timely access to resources is available. In addition, the quality of raw materials should be carefully evaluated because unexpected impurities may introduce unnecessary

Table 1.
Resource Requirements for Initial Protein Formulation Development

Resources	Requirement	Example
Purified protein	Representative of manufacturing process; sufficient quantity to cover dose bracket, formulation variables, and stress conditions; minimum complication by impurity (precipitation of impurity, degradation by impurity like proteolytic cleavage).	Purified bulk, sample from final purification process
Qualified excipients	Pharmaceutically acceptable quality, manufacturers with qualified production procedures and sufficient scale, specifications on critical impurities, quality that can be carried on to clinical studies and commercial distribution.	USP, Ph. Eur, JP
Access to fill finish facility	Capability to sterilize container/closure components; fill/finish under aseptic environment; head-space purge system; drying equipment	Sterile hood, filling machine, lyophilizer
Analytical instruments	Structural analyses; concentration determination, chromatographic analyses; electrophoresis; bioassays; other microcharacterizations.	CD, UV, fluorescence, HPLCs, mass-spectrometry, SDS-PAGE
Facility to accommodate stability studies	Controlled temperature, controlled light exposure, controlled relative humidity, devices to provide controlled agitation.	Freezer, refrigerator, incubator, light chamber, RH incubator, agitator

Samsung Bioepis Exhibit No. 1002
Page 7765

Table 2.
Examples of Information Useful for Designing Formulation Studies

Information	Examples
Clinical indication	Site of treatment (self-administration, office visit, hospital), methods of delivery, concomitant medication, competition.
Patient population	Age, strength, tolerability, capability to manipulate devices, sensitivity to excipient
Typical routes of delivery	Injectables (IV, SC, IM, IP, ICV, IT, IO), topical, inhalation, nasal, oral, etc.
Dose requirement	PK profile, frequency of dosing, variable vs. fixed dose, single-dose/multidose
Drug interaction	Co-administration with other drug, dilution or reconstitution with other solution; presence of undesirable compounds like reducing sugars, preservatives
Typical dosage forms	Liquid, lyophilized, spray-dried, aerosol by liquid or powder, other novel carrier; stability, physical properties, reconstitution art
Container/closure	Vial/stoppers, prefilled syringes, prefilled cartridges, dual-chamber cartridges, blister packages, product contact material, leacheates, breakage, light sensitivity, moisture penetration
Delivery device	Syringes, prefilled-syringes, pen injectors, auto-injectors, needle-free injectors, inhalation devices, infusion pumps

complications in the stability profile that is determined during formulation development and testing.

Useful Information for Designing Formulations

The configuration of a protein formulation is affected by how the drug will be used as a product. If such information is available when designing formulation studies, it is recommended to consider the limitations and challenges associated with each application. Examples of such information are listed in Table 2.

PREFORMULATION DEVELOPMENT

It is important to understand the critical properties of a protein before starting large studies to design and test the final formulation. Preformulation studies are designed to learn about the protein's susceptibility to a variety of pharmaceutically relevant stresses. The main objectives of preformulation research

Samsung Bioepis Exhibit No. 1002
Page 7766

Table 3.
Information Obtained from Pre-formulation Studies for Protein Pharmaceuticals

Characterization	Examples
Physical properties	Primary, secondary, tertiary and quaternary structures, solubility, viscosity, self-association, hydrophobicity, molecular weight, extinction coefficient, glycosylation, effects of ionic strength, etc.
Biological properties	Substrate or receptor affinity, in vitro activity model, *in vivo* preclinical model, etc.

include: general characterization of the product; investigation of potential stability issues; development of relevant analytical methods; establishment of a stability profile with stability-indicating assays; and identification of major formulation challenges. A summary of these issues will be presented in this section.

Characterization of Protein Pharmaceuticals

There is available in the literature extensive coverage of analytical methods and their principles for the characterization of proteins (e.g., Jones, 1993; Reubsaet et al., 1998; Herron et al., 1995). General points to consider when characterizing a protein as an active ingredient are listed in Table 3. As a protein's biological activity is dependent on its structure, significant emphasis has been given to structural properties and stability against various stresses.

Accelerated Stability Studies

In order to predict potential stability problems within a short period of time and to develop appropriate analytical methods, proteins are exposed to stronger-than-real stresses and various degradation products induced by the stresses are examined. The results obtained from these so-called "accelerated stability studies" might also be useful to predict the kinetics of the degradation processes under real handling conditions, when there are not sufficient real-time results available because of time and resource constraints. However, the accelerated stability study is not acceptable to determine expiry of the product, so it is best used to rank order the importance of different degradation pathways. Approaches and cautions in the extrapolation of data from accelerated stability testing to real-time stability and normal handling conditions are discussed in detail below.

Samsung Bioepis Exhibit No. 1002
Page 7767

Table 4.
Various Conditions Used to Accelerate Protein Degradation

Stresses	Routine ranges	Practical applications	Problems to monitor
Temperature	0–50°C	Storage, shipping, handling, delivery	Structural changes (precipitation, aggregation, recovery loss), solubility, increased reaction rates for all degradations
Light	>1.2 million lux hrs illumination, >200 watt hrs/square meter UV energy	Light exposure, container, package	Oxidation, cleavage
Freezing	Multiple freeze-thaw, liquid nitrogen freeze	Frozen storage, accidental freezing, lyophilization	Precipitation, aggregation, pH change, crystallization of excipients
Oxidation	Oxygen purge, peroxide spike	Storage, excipient stability, impurity	Oxidations, inactivation
Humidity	0–100% RH	Storage, container integrity, powder	Moisture content, moisture related degradations
Mechanical stresses	Vortex, agitation, shear-stress ($3000\,s^{-1}$)	Manufacturing, filling, shipping, handling, delivery	Precipitation, aggregation, recovery loss
Other denaturants	—	Impurities, pH, denaturing excipients	Precipitation, aggregation, recovery loss, structural changes

Conditions to accelerate various degradation reactions in protein products and potential problems to monitor are listed in Table 4. Proteins contain numerous amino acid side chains and delicate three-dimensional structures, which can be susceptible to different stresses. Therefore, it is important to test the protein under a variety of physical and chemical stresses in order to provide a good simulation of the degradation products that can be generated.

Development of Analytical Methods

A brief summary of typical analytical methods is presented in Table 5. It is essential to have a wide range of analytical methods available to identify and characterize degradation products. The analytical methods should be further selected and customized to accommodate the specific needs for each protein product.

Samsung Bioepis Exhibit No. 1002
Page 7768

Table 5.
Typical Methods Used to Characterize Proteins and Degradation Products

Methods	Examples	Applications
Column chromatography	HPLC, FPLC, low pressure LC; size-exclusion, reversed-phase, ion-exchange, hydrophobic, affinity columns; coupled with UV, fluorescence, RI, and other analytical instruments as detectors	Most physical and chemical degradations, excipient impurities, leacheates
Electrophoresis	SDS-PAGE, native PAGE, isoelectric focusing, capillary electrophoresis, etc.	Degradations with changes in size and/or charge
Spectroscopy	CD, fluorescence, FTIR, UV, Raman, NMR, etc.	Structural changes, chemical modifications of side groups
Thermal analysis	Differential scanning calorimetry, thermogravimetric analysis, thermomechanical analysis, etc.	Protein structure, lyophilized cake structure, powder characterizations
Light scattering/turbidity	Dynamic light scattering, other light scattering devices, turbidity, particle size determination, particle counter, etc.	Aggregation, precipitation, molecular weight determination
Other microcharacterization methods	Peptide mapping, peptide sequencing, amino acid analysis, mass spectrometry, other specific analyses for individual reactive groups	Identification of impurities and chemical degradation, analysis of complex proteins, e.g., antibody and glycoprotein

Evaluation of the Significance of Problems

As stated earlier, it would be ideal to have a pure protein in an absolutely stable formulation. In reality, however, scientists have to design formulation based on compromises that deal with several different potential problems. To make matters worse, the formulation needs to be recommended long before it is evaluated fully, because of typical aggressive timelines in the industry. In general, the formulation will have to be optimized based on assumptions and extrapolations of results obtained during a limited time. Therefore, it is important to utilize the given time efficiently, by collecting as much relevant information as possible for evaluating the significance of each problem.

Due to the marginal stability of proteins, it is possible to create rapidly a variety of degradation products during accelerated stability testing. However, not

Samsung Bioepis Exhibit No. 1002
Page 7769

all the degradation products that are observed will be significant under normal handling, shipping and storage conditions. Furthermore, the rank order of different degradation processes under accelerated stability testing will NOT be the same under practical handling conditions, because each reaction has a different temperature dependency, i.e., reaction order and activation energy. Another important thing to keep in mind when evaluating different degradation processes is the contribution to the pharmaceutical quality of the product. Critical degradation products should be designated not on the quantity obtained during accelerated stability studies, but on a comprehensive understanding of their contributions to the quality of the product.

Quantitative Assessment. Usually, the rate of each degradation reaction during real storage conditions (e.g., at 4–8°C) should be very slow. In order to predict the rate under real storage conditions, reaction rates obtained under accelerated stability conditions can be extrapolated by using the Arrhenius equation.

Predicting the correct reaction rate requires a proper understanding of the reaction order, because the amount of the degradation product does not linearly increase over time unless the reaction follows zero-order kinetics. One way to determine the reaction order is to calculate the linearity between the concentration of residual native protein and time. The reaction order can be calculated from:

$$dC/dt = kC^n \tag{1}$$

where, C is the protein concentration, t is the time, k is the reaction rate constant, n is the reaction order. For a zero order reaction $n = 0$ and $C_0 - C = k \cdot t$. For a first order reaction $n = 1$ and $\log C_0 - \log C = k \cdot t$. For a second order reaction $n = 2$ and $1/C - 1/C_0 = k \cdot t$. For each reaction order a characteristic transformation of concentration will show a linear relationship with time, e.g., log C will show a linear relationship with time for a first order reaction, whereas $1/C_0$ will be linear versus time for second order reactions.

After obtaining the reaction rate constants for different temperatures, the activation energy can be found by using with the Arrhenius equation.

$$k = A \cdot e^{(-Ea/RT)} \tag{2}$$

The activation energy is obtained by plotting (log k) vs (1/T) and determining the slope of the plot. Using the activation energy, the reaction rate constant for real storage condition, e.g., 2–8°C for refrigerated storage, can be estimated by extrapolation.

Care must be taken when extrapolating the rate constants because there are numerous cases when the Arrhenius relationship does not apply. The Arrhenius reaction applies only to irreversible reactions where the product is accumulated as a single quantifiable species. If the degradation product is the result of serial

Samsung Bioepis Exhibit No. 1002
Page 7770

reactions, the reaction order and rate constant will be determined only by the rate-limiting reaction at the testing condition. If the rate-limiting reaction changes or another significant variable is introduced at the extrapolated condition, then the extrapolated rate constant will be in error. Such complications routinely can be found in various degradation pathways that are affected by temperature-sensitive reactions such as structural changes, pH changes, by physical changes such as the glass transition of an amorphous phase in a lyophilized formulation and by changes in reactants like dissolved oxygen. For example, proteins have relatively high activation energies for structural changes. Thus, many reactions that are dependent on a major perturbation of the native protein structure tend to cause substantial damage under accelerated storage conditions, e.g., higher temperature. However, many of these reactions are not necessarily major problems when the product is stored at 2–8°C where the protein maintains its native conformation. In contrast, degradation reactions with lower activation energies, which might not be coupled to protein conformational changes (e.g., oxidation of surface methionine residues), tend to be much more problematic in the development of protein formulations.

Results from stresses (e.g., agitation) other than heat imposed in accelerated stability studies potentially could be extrapolated in a similar way to storage and actual handing conditions. The major challenge is the ability to assess quantitatively the magnitude of the stress so that the stress-stability relationship can be established. To date there are not published guidelines for such quantitation.

Qualitative Assessment. Not all of the degradation products are equivalent in terms of their contribution to the pharmaceutical quality of the protein product. Therefore, a qualitative assessment of each degradation product is important when weighing their significance. Regardless of their quantity, some degradation products are generally less acceptable than others. If the degradation product comprises the safety of the product, then it should be considered less acceptable. For example, even at levels of a few percent or less of the total protein population, non-native aggregates can cause adverse reactions in patients such as immune responses and even anaphylactic shock. Also, if the degradation reaction results in the inactivation of protein, it needs to be considered more important than other degradation reactions that do not affect the activity. For example, certain chemical changes (e.g., deamidation) may not alter the activity of a given product, whereas other reactions (e.g., oxidation) may render that product inactive. The relative impact of each degradation product on safety and efficacy cannot be predicted and must be determined for each protein therapeutic.

Information useful for the qualitative evaluation of degradation products includes the identity, clinical and preclinical experiences with the degradation product, biological activity, stability and potential side effects. Regulatory documents also provide clear guidance about what assessment is necessary for protein degradation products (Appendix, Regulatory Documents 2,3). The criteria for

Samsung Bioepis Exhibit No. 1002
Page 7771

identification, reporting and qualification of degradation products are set up based on the total patient exposure and percentage of the degradation products (Appendix, Regulatory Document 3). When certain degradation products cannot be prevented from forming in the formulation, their inclusion in the product must be qualified, which may require that additional information on safety and efficacy be obtained as recommended in the regulatory guidelines (Appendix, Regulatory Documents 2,3). Obtaining this information requires a significant amount of additional work, which may include clinical studies.

FORMULATION DEVELOPMENT

As discussed above, the critical parameters affecting the pharmaceutical quality of a protein therapeutic are defined during the preformulation studies. In formulation development, the effects of formulation variables on the defined critical parameters are examined to optimize protein stability. Which variables are most important depends of the formulation type chosen. For example, resistance to agitation and/or accidental freezing is a critical property for an aqueous formulation, which would not be a concern for a lyophilized formulation. Similarly, choosing excipients that provide a glassy matrix to stabilize the protein is only a concern for dried formulations.

Formulation Options for Protein Pharmaceuticals

Different types of formulations need to be developed based on clinical needs, patient compliance, delivery method, stability of the drug, storage and distribution, and market competitiveness. Having a clear plan about what type of formulation is desired will allow one to design better formulation studies. Liquid formulations have been generally preferred due to the convenience of manufacturing and use. However, protein drugs may not be stable enough to be handled as a liquid formulation. Dried formulations (e.g., lyophilized) or suspension formulations (e.g., insulin zinc suspension) have been successfully used to overcome stability problems. In addition, specific applications and delivery may demand the appropriate type of formulation, e.g., a spray-dried powder for pulmonary delivery. As details of the stability issues are discussed in other chapters in this book, only practical issues regarding these different types of formulations will be summarized here.

Liquid Formulations. It is important to understand that developing conditions to keep proteins stable in a liquid form for a pharmaceutically relevant

Samsung Bioepis Exhibit No. 1002
Page 7772

storage time (e.g., two years) is not a simple task. For most proteins, including relatively stable ones, at least some degradation should be expected even during refrigerated storage. Unless there is strong evidence supporting that the protein remains stable for two years and the evidence is supported by a broad spectrum of analytical methods, one needs to be very careful about the final decision to market a liquid formulation. Physical or chemical changes that have low activation energy should be monitored especially carefully under real storage conditions. If there is a degradation product that can significantly affect the pharmaceutical quality at its minimum concentrations (e.g., formation of particulates) great care must be taken to assure that such a product does reach unacceptable levels during real-time storage. If long-term storage studies are not implemented until late in the product development process, there is a risk that problems due to unacceptable levels of degradation products will not be discovered in time to test alternative formulations. In addition to stability during storage, temporary exposure to temperatures outside the recommended conditions should also be tested because it can affect the quality of the drug. Also, results obtained from multiple freeze-thawing cycles will be useful to determine if the formulation can accommodate unexpected freezing during distribution and storage. Likewise, sensitivity to agitation or surface denaturation needs to be understood to support formulation choices as well as shipping and handling guidelines. For formulations containing low protein concentration, special attention is required to avoid impurity-related stability issues (e.g., oxidation fostered by metal contaminants in excipients).

Solid Dosage Forms. Recent improvements in devices designed for easier use of lyophilized products, e.g., dual chamber syringes, dual chamber cartridges and convenient reconstitution devices, have helped pharmaceutical industries to develop lyophilized products without too many concerns surrounding patient compliance issues. Because lyophilized products have less stability-related issues and have a much greater potential tolerance for room-temperature storage, many biopharmaceutical industries consider the lyophilized formulation as a default option. In fact, lyophilization has been widely utilized to overcome various stability issues of labile proteins. However, it is important to note that lyophilization can present its own challenges, particularly in designing appropriate formulations and economic cycles. Detailed discussion about the rational design of stable lyophilized formulations is available in another chapter in this book.

In addition to the required stability, a successful lyophilized formulation should also have the desired physical properties of the dried powder, e.g., ruggedness of the cake and the maintenance of the physical states for each of the ingredients. For example, crystallization of an initially amorphous excipient during storage can lead to unacceptable degradation of the protein product and loss of cake structure. Furthermore, optimal water content for protein stability should be studied and controlled during storage. It is preferable to define an acceptable

Samsung Bioepis Exhibit No. 1002
Page 7773

range of water contents to provide flexibility in the manufacturing process. To assure that the appropriate water content is maintain, the integrity of container/packaging should be monitored in a high relative humidity environment.

The identity and volume of the reconstitution medium should also be clearly defined, because the use of injectable fluids other than the recommended one may compromise the quality of the product (e.g., solution tonicity or protein stability). Reconstitution should be convenient and rapid, and the stability of the reconstituted solution during handling and delivery needs to be demonstrated.

Only recently have spray-dried formulations become commonly used with the advent of novel protein delivery technologies. For example, spray-dried powders have been useful for developing products that require uniform particle size (e.g., pulmonary inhalation for systemic delivery). Similar issues to the lyophilized formulation apply to the spray-dried formulation, and the details of development of spray-dried formulations are presented in another chapter in this book. In addition to protein stability, physical properties pertinent to powder processing and powder stability (e.g., flowablity, hygroscopicity, agglomeration, density and crystallization of excipients) need to be well characterized.

Single Dose and Multidose Forms. Most protein pharmaceuticals are marketed as single dose forms. However, multidose formulations are useful when the dose needs to be split (e.g., dose titration or dose combination). Multidose formulations are distinguished by the presence of preservatives in the formulation, which prevent microbial contamination and/or growth during multiple disruptions of container closure integrity. In general, the addition of preservative(s), regardless of the preservative used, significantly changes the stability profiles of proteins (Maa and Hsu, 1996; Fransson et al., 1997; Lam et al., 1997). In some extreme cases, visible precipitation and aggregation have been reported. Therefore, the effect of various preservatives on the stability of protein should be carefully examined. Other experiments required to qualify a multidose formulations include the preservative effectiveness test and the stopper self-sealing test. Detailed procedures and specification are available from the USP (U.S. Pharmacopeia), the Ph. Eur. (European Pharmacopeia) and the JP (Japan Pharmacopeia).

Desired properties of the ideal preservative include: effectiveness at low concentration against a wide variety of organisms; chemical stability; solubility; compatibility with the protein drug, excipients and auxiliary agents; free from objectionable odor, taste, color and stinging; and non-toxic and non-sensitizing both internally and externally at the required concentration. Also, it must not absorb, penetrate, or interact with containers or closures (Thompson, 1998). For further details on preservatives, readers are referred to Regulatory Document 4 in the Appendix and Thompson (1988). Examples of typical preservatives used for parenteral protein pharmaceuticals are benzyl alcohol, phenol, *m*-cresol and benzalkonium chloride.

Samsung Bioepis Exhibit No. 1002
Page 7774

Typical Protein Stability Problems: Causes and Solutions

Table 6 summarizes typical stability problems observed during protein formulation development and potential methods to solve each problem. The list does not represent the complexity of multiple problems that can be experienced with a given protein. Formulation research should be designed to handle each protein based on its unique stability profile.

Optimization of Formulation Variables

Overview of the Process. The optimization of formulation variables for product stability is the most critical part of protein formulation development. Various formulation excipients and buffers (Table 7) can be utilized and must, therefore, be chosen to maximize the pharmaceutical quality of the product (i.e., stability and activity) without introducing significant side effects. Among the listed formulation variables, the most powerful one is pH. Problems associated with the physical properties of a protein, e.g., precipitation due to solubility and/or stability, are generally very difficult to manage by other formulation means. Optimization of pH is a simple but very useful solution for such problems (Kolvenbach et al., 1997). Most chemical reactions also are affected by pH, e.g.,

Table 6.
Typical Stability Problems Observed in Protein Pharmaceuticals

Problems	Potential causes	Possible solutions
Non-covalent aggregation	Solubility, structural changes, heat, shear, surface, denaturants, impurities	pH, ionic additives, amino acids, surfactants, protein concentration, raw material purity
Covalent aggregation	Disulfide scrambling, other unknown mechanisms	pH, inhibit non-covalent aggregation
Deamidation	pH < 5.0 or pH > 6.0	pH optimization
Cyclic imide	pH around 5	pH optimization
Cleavages	Protease impurity, other unknown mechanisms	pH, product purity, inhibitors
Oxidation	Active oxygen species, free radicals, metals, light, impurity	Excipient purity, free-radical scavenger, active oxygen scavengers
Surface denaturation, adsorption	Low protein concentration, specific affinity, protein hydrophobicity	Surfactants, protein concentration, pH

Samsung Bioepis Exhibit No. 1002
Page 7775

Table 7.
Important Components of Protein Formulations

Formulation Variables	Desired attributes	Examples
pH	Provides good physical properties of protein, minimize degradations	
Stabilizer	Inhibit degradations, effective at low concentrations	Surfactants, sugars, salts, antioxidants
Solubilizer	Improve the solubility, effective at low concentrations	Salts, amino acids, surfactants
Buffer	Good buffering capacity, stable to temperature change, stable to freezing, good safety record	Phosphate, acetate, histidine, glutamate
Tonicity modifier; bulking agent	Inert, good safety record	Sodium chloride, sorbitol, mannitol, glycine

deamidation, cyclic imide formation, disulfide scrambling, peptide bond cleavage, and oxidation (see reviews listed above). Other functional excipients should be also carefully evaluated for the benefit of the product (e.g., use of sucrose to stabilize protein during lyophilization and storage in the dried solid).

In addition to their intended use, excipient candidates should be also qualified as appropriate pharmaceutical ingredients. Generally, it is preferred to select excipients that have been used in marketed products with a relevant route of delivery. Further, it is preferable if these excipients have been used with similar frequency of dosing, history of chronic use and similar patient populations. Otherwise, the approval and safety of the excipients need to be carefully examined. If the excipient is considered safe with a solid scientific basis or has a proven clinical safety record, it can be considered equivalent to approved excipients. The list of excipients used for parenteral pharmaceuticals is available in the literature (Powell et al., 1998; Nema et al., 1997; Appendix Regulatory Document 5). When it is necessary to introduce other excipients with minimum safety records, a significant risk associated with the excipients will be added to the product development and additional pre-clinical and clinical studies may be needed.

Another important requirement in qualifying an excipient is the purity of the raw material. Depending on the historical use as a pharmaceutical ingredient, several different pharmaceutical grades are available, e.g., USP (U.S. Pharmacopeia), Ph. Eur. (European Pharmacopeia) and JP (Japan Pharmacopeia). These pharmaceutical grade materials should be considered as a primary resource, but the quality provided may not be good enough for specific product development. For example, significant stability problems can be found with some impurities

Samsung Bioepis Exhibit No. 1002
Page 7776

even at concentrations below their specification, e.g., metal ions, peroxides, proteases and reducing sugars. These problems are more prominent in low protein concentration products due to a high impurity-protein ratio, although problems like visible precipitation of the protein may be independent of protein concentration. If adjustment in the existing specification is necessary, it is critical to look into the availability of GMP quality raw materials with modified specifications as early as possible, once the potential problem is identified.

The use of excipients derived from animals (e.g., Tweens) or humans (e.g., human serum albumin) should be avoided if possible due to the risk associated with transmissible diseases like bovine spongiform encephalopathy, Creutzfeldt-Jakob Disease, hepatitis virus and HIV. Numerous regulatory guidelines have been issued to discourage the use of animal/human-derived excipients (Appendix Regulatory Documents 6,7,8). When animal-derived excipients have to be included in the product, the manufacturer will need to demonstrate that their selection is fully justified.

Design of the Study. A design for the formulation optimization study should be in place before commencing the work. A typical study protocol will include the following information:

- Study title
- Study objective
- Source and quality of drug substance and excipients
- Material preparation
- Formulation matrix
  - Formulation variables
  - Protein concentrations or bracket
- Analytical methods
- Storage conditions (temperature, light, humidity)
- Additional sample handling conditions (temporary exposure to stresses, container orientation, etc.)
- Sampling schedule and expected duration of the study
- Plan for data analysis and report

The study can be more efficiently designed by utilizing experimental design software packages, which will help to minimize the resources required for sample preparation, analyses, and data analysis.

Necessary Studies for Formulation Development

Storage Stability Study. Documenting that the formulation will keep the protein stable until the desired expiry can be the most time-consuming part of

Samsung Bioepis Exhibit No. 1002
Page 7777

formulation development. The expiry requirements are determined by distribution not regulatory requirements. One year is probably too short for effective manufacturing and distribution through normal channels. In general, a shelf life of 18 month is considered acceptable for commercialization. Results obtained from accelerated stability studies are useful for predicting potential degradation products and appropriate analytical methods, but whatever the actual shelf life, it must be supported by sufficient real-time storage data to obtain regulatory approval. Thus, it is important to establish a final formulation and start the real-time storage studies as early as possible during product development.

Process Development. Formulations that can be prepared on a small scale without experiencing any problems may encounter significant problems during the scale-up of the process. For example, mixing solutions in a large stainless steel tank, pumping solutions through stainless steel tubing, filtration and filling through a high-speed filling machine can introduce unexpected stresses to the protein. An increase in the formation of particulates, along with a loss of proteins due to surface adsorption and aggregation, has routinely been observed. It is important to expose the formulation to equivalent stresses and make sure that no formulation adjustment is necessary to accommodate the manufacturing processes. Again, this testing should be done as early in the development process as possible.

Transportation, Handling and Delivery Study. Unexpected environmental changes can be encountered during the distribution and handling of products (e.g., accidental freezing, exposure to temperatures different from the recommended conditions, vigorous agitation, etc). It is critical to develop the formulation with these stresses in mind because they can compromise the quality of the product.

Also, during administration to the patient, proteins can be exposed to different types of stresses introduced by the device and the routes of delivery. Examples include incompatibility with the delivery device (e.g., protein aggregation induced by exposure to tubing surfaces) and/or concomitant medication (e.g., protein aggregation induced by co-administered antibiotics).

Preclinical and Clinical Studies. Results documenting the maintenance of the biophysical and biochemical properties of the protein are essential for a final formulation decision. Before finalizing the formulation, it is also important to confirm that it does not affect critical *in vivo* biological properties of the protein (e.g., activity, pharmacokinetic profile, and toxicity profile). Maintenance of a protein's biophysical properties can be examined using various structural analyses (e.g., circular dichroism, fluorescence and infrared spectroscopies, etc.). It is possible to determine the biochemical equivalence of the protein pharmaceutical by *in vitro* activity and/or preclinical *in vivo* bioassays. Results supporting the toxicity profile of the formulation can be generated by both preclinical and clinical studies.

Samsung Bioepis Exhibit No. 1002
Page 7778

Strategies to Overcome Difficult Formulation Problems

Occasionally, problems are encountered that are difficult to overcome with conventional formulation approaches. It is possible to conduct additional studies to confirm that the problem is pharmaceutically acceptable. On the other hand, unique formulation approaches can be introduced to address specific problems. Examples of this approach are provided below.

Qualification of Degradation Products. If decreasing the amount of degradation product below the specified threshold is not feasible, then the degradation product can be defined as a drug substance by demonstrating that it does not affect the pharmaceutical quality of the drug product, i.e., safety and efficacy (Appendix, Regulatory Document 1). If there is not sufficient information available, additional qualification studies recommended in the regulatory guidance can be carried out (see the discussion above). The experience of having these degradation products in the materials used for clinical studies can sometimes be very useful because the results obtained can provide useful insight into the clinical implication of the degradation products.

Site-directed Mutagenesis to Improve Properties. Some problems related to the intrinsic properties of a protein cannot be overcome unless a change in the sequence is introduced. After careful research is carried out to identify the problematic region or residue, the protein can be engineered for better physical and/or chemical properties. For serious problems like precipitation due to insolubility or poor stability, changes in protein sequence have been proven effective for improving physical properties (Murby et al., 1995; Roig and Kennedy, 1995).

Chemical Modifications. The physical properties of proteins can be improved by modifying problematic amino acid side chains with small compounds or large polymers (e.g., attachment of polyethylene glycol) (Fagain, 1995; Guerra et al., 1998; Francis et al., 1998). In addition, there are other desirable improvements that can be achieved by the conjugation chemistry (e.g., enhanced pharmacokinetic profiles, reduced immunogenecity, enhanced adsorption, etc.).

Unconventional Dosage Forms. Some protein stability problems can be resolved by introducing novel approaches in the formulation. Examples include suspension formulations (Defelippis et al., 1998), microencapsulation with cyclodextrin (Brewster et al., 1991), suspension of dry-powder in non-aqueous vehicle (Knepp et al., 1998), and use of non-aqueous vehicles with hydrophobic ion-pairing (Manning et al., 1995).

Samsung Bioepis Exhibit No. 1002
Page 7779

FORMULATION IN COMMERCIAL PRODUCT DEVELOPMENT

Critical Formulation Decisions During Pharmaceutical Development

A brief summary of the important phases of a commercial product development is shown in Figure 1. Ideally, a stable formulation would be available during the early discovery research period to ensure that the protein is in its active and stable form during critical feasibility studies. However, development of a stable formulation for each drug candidate is not practically possible because such an approach would take too much time and too many resources. Generally, formulation development starts after the decision is made to start clinical trials. A

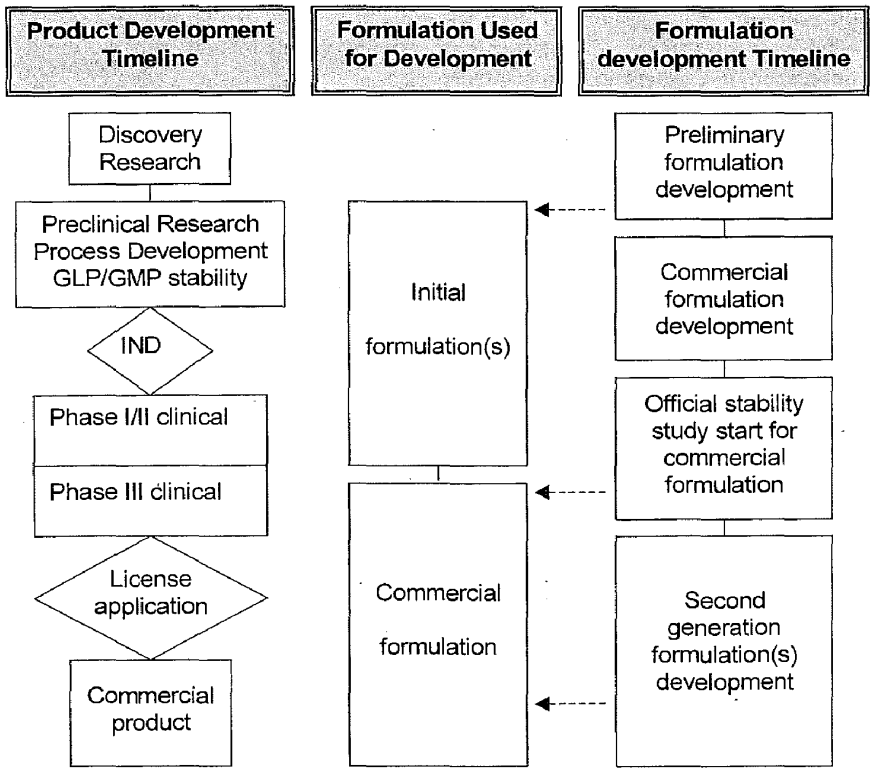


Figure 1. Diagram showing commercial formulation development process.

Samsung Bioepis Exhibit No. 1002
Page 7780

reliable formulation is required to support various preclinical and clinical studies. The formulation can be further improved later to satisfy the clinical, marketing, and regulatory needs. Although formulation development does not necessarily appear rate-limiting during this improvement period, it is important to understand that changing a formulation may require much more supporting work, including additional clinical trials, which can take years.

Formulation for Early Preclinical and Clinical Studies

When a promising protein drug candidate is identified from preliminary feasibility studies, a decision will be made to introduce the drug to clinical studies. In order to obtain regulatory approval to initiate the clinical trials, it is required to demonstrate safety, manufacturing capability, stability of the drug and reproducibility in studying the drug. A sufficiently stable formulation is necessary at this stage because it is important to maintain the quantity and quality of the protein for all research studies from which results will be used for registration of the product with regulatory agencies. Therefore, formulation development becomes a rate-limiting process at this early stage. Formulations can be stored frozen or lyophilized for this purpose. At this stage in product development, the shelf-life requirement is determined by the logistics of supplying drug for clinical trials.

Commercial Formulation

Commercially viable and market competitive formulations have some common features. Most of all, the formulation should maintain the safety and efficacy profile of the protein drug during all the handling and uses specified on the label. Since commercial distribution channels are not equipped for frozen products, shipping and storage at refrigerated temperature or higher are required. Sufficient shelf life needs to be determined under conditions to which the product will be exposed in the commercial distribution system. Various systematic studies need to be carried out to comply with regulatory requirements for registration. It takes 1–2 years to collect all of these results, so the commercial formulation is developed while preclinical studies or early clinical trials (with the preliminary formulation) are in progress. If possible, the commercial formulation should be introduced before the pivotal clinical trial because clinical experience is the most effective way to confirm the safety and efficacy aspects of the formulation. In addition, formulation changes after this point may introduce formidable challenges to the clinical program and to obtaining regulatory approval.

Samsung Bioepis Exhibit No. 1002
Page 7781

Regulatory Issues in Formulation Development

Incorporating regulatory guidelines into the formulation development is not only useful to develop high quality pharmaceutical formulations for maximum benefit to the patients, but also critical to prepare complete documents necessary for regulatory approval for commercialization of the drug. Readers who wish to obtain a comprehensive understanding of the guidelines are referred to the relevant documents published by regulatory agencies. In this section, a brief summary of the guidelines related to the formulation issues is provided.

Guidelines for Stability Studies. Comprehensive guidelines can be obtained from regulatory agencies (Appendix, Regulatory Documents 9–12) and review articles in the literature (Grimm, 1998; Kommanaboyina and Rhodes, 1999; Matthews, 1999). These guidelines generally cover the formal stability studies for bulk drug material, in-process samples and final formulated product. Although some issues may not be relevant for the earlier formulation development work, these guidelines provide key elements for designing stability studies. The guidelines provide information such as what types of experiments should be included in the study protocol, how to propose a stability-indicating profile, and what analytical results are needed to define the purity of the product and the molecular characteristics of degradation products. Another important piece of information provided in the guidelines is the definition of proper experimental conditions, e.g., definition of storage temperature, humidity, light strength, and accelerated and stressed conditions. The guidelines also provide the official protocols to evaluate the results obtained from stability studies.

Results Required to Apply for Regulatory License for a Drug Product. Detailed information regarding the formulation is presented at the Development Pharmaceutics section in the CMC section of regulatory applications (Appendix, Regulatory Docoments 13–16). All of the essential information required to apply for regulatory approval of protein formulations can be found in Note for Guidance on Development Pharmaceutics (Appendix Regulatory Document 17) and Development of Pharmaceutics for Biotechnological and Biological Products (Appendix, Regulatory Document 8). It is important to note that this is currently a European requirement although the international harmonization process may eventually introduce it to U.S. regulatory requirements. A brief summary of the important formulation information is shown in Table 8.

Results Required to File Formulation Amendments. A formulation change can introduce substantial potential adverse effects on the identity, strength, quality, purity or potency of the product as related to the safety or effectiveness of the product. For this reason, it is required that a supplement to the approved license application be submitted. This supplement should include a detailed description of the proposed change. It should also include methods and results for the studies performed to evaluate the effect of the change on the

Samsung Bioepis Exhibit No. 1002
Page 7782

Table 8.
Summary of Information Included in Regulatory Applications

	General information	Comments specific to proteins
Active substance (protein)	Compatibility with excipients and other combined products, and physicochemical characteristics	Structural elements responsible for the biological activity; good coverage of degradations occurring during storage, manufacturing, and delivery.
Excipients	Function of each excipients, justification for their inclusion, and compatibility with other excipients, choice of quality	
Formulated products	Overage, physicochemical parameters, components with appropriate results supporting intended purpose; compatibility with diluent, device, and other drugs in contact before delivery; critical physical properties	Stability of structure in terms of biological activity; formulation optimization for both manufacturing process and stability
Packaging material	Integrity of container and closure during storage, reconstitution, admixture, dilution; sorption to container, leaching, and dose reproducibility	Adsorption, denaturation at the interface, and aggregation of the surface-denatured protein; reconstitution of dry-powder formulation
Manufacturing process	Manufacturing process for the preparation of formulation and its justification, appropriate method of sterilization and justification	Results to support the quality and stability of the protein during the manufacturing process; membrane filtration under aseptic conditions sufficient

product's identity, strength, quality, purity, and potency of the product, as related to the product's safety or effectiveness (Appendix, Regulatory Documents 18–20). When the degradation profile is changed qualitatively or quantitatively, it is recommended to follow the impurity-related guidelines discussed above (Appendix, Regulatory Documents 2,3). The manufacturer must obtain approval of the supplement by FDA prior to distribution of the product made using the change. In general, the following studies (Table 9) can be carried out to confirm

Table 9.
Studies Needed to Support Change in Formulation

Research requirements	Supporting results
Purity	Stability, compatibility, Structural analyses
Potency	*In vitro*/*in vivo* bioassays, preclinical and/or clinical pharmacokinetic comparability , clinical efficacy
Safety	Preclinical safety, clinical safety

Samsung Bioepis Exhibit No. 1002
Page 7783

that the change in the formulation will not affect the safety and effectiveness c the product. It is recommended to consult the regulatory representative to fin out how much additional information may be needed for the approval of th changed formulation.

APPENDIX: LIST OF REGULATORY DOCUMENTS

1. International Conference on Harmonization: Guidance on specifications: Te procedures and acceptance criteria for Biotechnological/Biological Produc Federal Register: August 18, Volume 64, Number 159, pp. 44928–449 (1999).
2. International Conference on Harmonization: Impurities in New Drug Prc ucts. FDA Q3B (11/97).
3. Guidance for Industry. ANDAs: Impurities in Drug Products. Draft guidan FDA (12/98).
4. Note for Guidance on Inclusion of Antioxidants and Antimicrobial Pres vatives in Medicinal Products (CPMP/CVMP/QWP/115/95) EMEA (7/9
5. Inactive ingredient guide: inactive ingredients for currently marketed dı products. (1996) FOI Services, Inc. Rockville, MD.
6. Note for guidance on minimizing the risk of transmitting animal spongifc encephalopathy agents via medicinal products. EMEA (4/99).
7. Note for guidance on plasma-derived medicinal products. EMEA (7/98).
8. Development Pharmaceutics for Biotechnological and Biological Produ Annex to Note for Guidance on Development Pharmaceutics (CPM QWP/155/96). EMEA (10/1999).
9. International Conference on Harmonization: Stability testing of new d substances and products. Federal Register, Sept. 22, Volume 59, Number 1 pp. 48754–48759 (1994).
10. International Conference on Harmonization: Final guideline on stab testing of biotechnological/biological products. Federal Register, July Volume 61, Number133, pp. 36466–36469 (1996).
11. Guideline for Industry: Stability testing for drug substances and drug p ucts: draft guidances. FDA, (6/98).
12. International Conference on Harmonization: Guidelines for the photost ity testing of new drug substances and products. Federal Register, May Volume 62, Number. 95, pp. 27115–2712 (1997).
13. Guideline for Submitting Documentation for the Stability of Human D and Biologics FDA. (2/87).
14. Guidance for Industry. For the Submission of Chemistry, Manufacturing Controls and Establishment Description Information for Human Pla

Samsung Bioepis Exhibit No. 1002
Page 7784

Derived Biological Products, Animal Plasma or Serum-Derived Products. FDA (2/99).
15. Guidance for Industry. INDs for Phase 2 and 3 Studies of Drugs, Including Specified Therapeutic Biotechnology-Derived Products Chemistry, Manufacturing, and Controls Content and Format (Draft guidance) FDA (2/99).
16. Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-Derived Products, FDA (11/95).
17. Note for Guidance on Development of Pharmaceutics (CPMP/BWP/328/99). EMEA (01/1998).
18. Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-Derived Products, April, 1996.
19. Guidance for Industry: Changes to an Approved Application. For Specified Biotechnology and Specified Synthetic Biological Products, 21 CFR 601.12, 314.70; July 24, 1997, Vol 62. No. 142.
20. Guidance for Industry: Changes to an Approved Application: Specified Biotechnology and Specified Synthetic Biological Products; July 1997.

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Samsung Bioepis Exhibit No. 1002
Page 7787

5

Rational Design of Stable Lyophilized Protein Formulations: Theory and Practice

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INTRODUCTION

For ease of preparation and cost containment by the manufacturer, and ease of handling by the end user, an aqueous therapeutic protein formulation usually is preferred. However, with many proteins it is not possible—especially considering the time constraints for product development—to develop sufficiently stable aqueous formulations. Unacceptable denaturation and aggregation can be induced readily by the numerous stresses to which a protein in aqueous solution is sensitive; e.g., heating, agitation, freezing, pH changes, and exposure to interfaces or denaturants (Arakawa et al., 1993; Cleland et al., 1993; Brange, 2000; Bummer

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Samsung Bioepis Exhibit No. 1002
Page 7788

and Koppenol, 2000). Furthermore, even under conditions that thermodynamically greatly favor the native state of proteins, aggregation can arise during months of storage in aqueous solution (e.g., Gu et al., 1991; Arakawa et al., 1993; Chen et al., 1994; Volkin and Middaugh, 1996; Chang et al., 1996a). In addition, several chemical degradation pathways (e.g., hydrolysis and deamidation) are mediated by water. In aqueous formulations, the rates of these and other (e.g., oxidation) chemical degradation reactions can be unacceptably rapid on the time scale of storage (e.g., 18–24 months) for pharmaceutical products (Manning et al., 1989; Cleland et al., 1993; Goolcharran et al., 2000; Bummer and Koppenol 2000).

In contrast, a properly lyophilized formulation can maintain adequate physical and chemical stability of the protein during shipping and long-term storage even at ambient temperatures. As will be outlined in this chapter, developing stable lyophilized protein formulations should be a rational, straightforward process, which for most proteins should be rapid. With liquid formulation development, it may only be possible to obtain adequate protein stability after lengthy studies. Furthermore, sometimes there are conflicting conditions (e.g., pH) needed to slow sufficiently multiple degradation pathways in aqueous solution. Considering these issues plus the fact that formulation scientists now have to deal with numerous proteins and/or variants of a given protein, lyophilization should be considered as a primary mode for product development. Only if a parallel effort to develop an aqueous formulation is successful, will a final lyophilized product not be needed.

Rapid formulation development has important financial ramifications. A drug product has a finite patent life, during which time the company has an exclusive market. Considering that even a moderately successful drug product has annual sales of hundreds of millions of dollars, potentially millions of dollars in sales are lost for each day of delay in bringing a product to market. Unfortunately there are often delays because the formulations designed during early stage development and clinical trials (e.g., frozen) were not adequate for the final product. With a rational approach to formulation development, pharmaceutical scientists and process engineers can minimize the risk of this problem and the time needed to obtain a successful, final formulation. The success of such efforts depends on frank, open communication between the groups involved. For example, it is critical that the formulation scientists learn from the process engineers the issues for large-scale lyophilization runs, which are usually conducted in units that do not have the capacity to match processing parameters obtained in a small-scale research lyophilizers.

Despite the best efforts of the scientists and engineers, all too often delays in formulation development arise because sufficient resources are not invested in product development. For example, sometimes, purchase of essential equipmen

Samsung Bioepis Exhibit No. 1002
Page 7789

(e.g., a differential scanning calorimeter), which costs a minute fraction of a day's sale of product, is not allowed. To avoid unnecessary delays in product launch, which can have disastrous consequences for the company and for patients, it is essential that companies appreciate that product development is ultimately a key limiting factor in getting a therapeutic to market. Hence, development efforts need to be as well funded as the usually much more visible drug discovery research programs. If a product is not stable, it will not be marketed, no matter how dramatic an impact it can have on human health and the financial status of the company.

MINIMAL CRITERIA FOR A SUCCESSFUL LYOPHILIZED FORMULATION

Research over the past several years has demonstrated that five criteria define the minimal conditions necessary for a successful lyophilized protein formulation (Table 1).

The first four criteria can be met with use of the appropriate excipients and lyophilization cycle design. For information on the proper design of lyophilization cycles, the reader is directed to the numerous previous reviews in this area (Franks, 1990; Pikal, 1990; Nail and Gatlin, 1993; Gatlin and Nail, 1994; Carpenter and Chang, 1996; Rey and May, 1999; Cappola, 2000). For the current chapter we will only consider cycle design in terms of the interplay between formulation physical properties (e.g., collapse temperature) and process parameters (see below). The last criterion listed in Table 1 requires insight into the unique physicochemical properties of each therapeutic protein, which will be explained in more detail below. We will discuss in turn why each of these criteria is important. Then we will present an explanation of how to design rationally a formulation to meet these criteria.

Table 1.
Minimal Criteria for a Successful Lyophilized Protein Formulation

1. Protein unfolding during freezing and drying is inhibited.
2. The glass transition temperature of the product exceeds the planned storage temperature (e.g., $T_g > 30°C$).
3. The water content is relatively low (e.g., 1% by mass).
4. A strong, elegant cake structure is obtained (i.e., collapse and meltback are avoided).
5. Steps are taken to minimize specific routes of protein chemical degradation (e.g., product vials are sealed under nitrogen to reduce the rate of methionine oxidation).

Samsung Bioepis Exhibit No. 1002
Page 7790

Inhibition of Lyophilization-Induced Protein Unfolding

The stresses of freezing and drying cause protein unfolding, and the formulation must be designed to inhibit unfolding at each step (Prestrelski et al., 1993a,b; Carpenter et al., 1993; Prestrelski et al., 1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Chen et al., 1999; Bell, 1999; Carrasquillo et al., 2000). Even if the formulation excipients and/or intrinsic thermodynamic stability of the protein prevent denaturation during freezing, unfolding can arise during subsequent drying (Carpenter et al., 1993; Prestrelski et al., 1993b; Allison et al., 1998; Carrasquillo et al., 2000). Conversely, once a protein unfolds during freezing, it will not regain native structure during dehydration.

For many proteins, unfolding during lyophilization leads to clinically unacceptable, non-native aggregates, even when samples are rehydrated immediately after lyophilization (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a, 1999; Costantino et al., 1998). Aggregates are not necessarily formed during freezing and drying. Rather, during rehydration refolding of structurally perturbed protein molecules competes with formation of non-native protein aggregates (Prestrelski et al., 1993a). Aggregation can be minimized by including stabilizing excipients (e.g., sucrose or trehalose) in the formulation to inhibit lyophilization-induced unfolding (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a, 1999; Costantino et al., 1998). Furthermore, fostering refolding during rehydration (e.g., with surfactants) can reduce aggregation (Chang et al., 1996c; Zhang et al., 1995, 1996).

In addition to minimizing protein aggregation during lyophilization/rehydration, maximizing retention of native protein structure in the dried solid is essential for optimizing long-term storage stability (Prestrelski et al., 1995; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Allison et al., 2000; Cleland et al., 2001). Both chemical and physical degradation in the dried solid can be accelerated if protein unfolding is not inhibited during lyophilization. With chemical degradation, a non-native structure may provide an environment conducive to covalent modification of one or more residues. For example, exposure of a methionine, which is normally buried deep in the interior of the native protein, on the surface of an unfolded dried protein may foster oxidation. Increased levels of aggregates noted after storage and rehydration of unfolded proteins could be due to formation of non-native intermolecular contacts within the dried solid, perturbation of refolding during rehydration because of chemical degradation, and/or other undefined processes.

Infrared spectroscopy has been used routinely to compare the secondary structures of a protein in lyophilized formulations to that of the native protein in aqueous solution (Prestrelski et al., 1993a,b; Prestrelski et al., 1995; Dong et al.,

Samsung Bioepis Exhibit No. 1002
Page 7791

1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Carpenter et al., 1998; Chen et al., 1999; Carrasquillo et al., 2000). This method should be considered essential in the development of stable lyophilized formulations, because it allows one to assess rapidly the effectiveness of formulations at inhibiting protein unfolding. Technical details about how to employ infrared spectroscopy to design stable lyophilized protein formulations can be found in the papers cited above.

Storage at Temperatures Below Formulation Glass Transition Temperature

In the dried powder, the protein is a component of an amorphous phase that includes amorphous excipients and water. If this glassy matrix is held below its characteristic glass transition temperature (T_g), the rate of diffusion-controlled reactions, including protein unfolding/aggregation and chemical degradation, are greatly reduced, relative to rates noted at temperatures $>T_g$ (Roy et al., 1991; Franks, 1990; Franks et al., 1991; Pikal, 1994, 1999). T_g can be determined with differential scanning calorimetry (DSC) or other thermal scanning methods (Nail and Gatlin, 1993; Chang and Randall, 1992; Craig and Royall, 1998; Verdonck et al., 1999).

Obtaining a formulation T_g in excess of the planned storage temperature (e.g., room temperature) is absolutely essential for optimal protein stability (e.g., Franks et al., 1991; Pikal, 1994, 1999; Carpenter and Chang, 1996; Duddu and Dal Monte, 1997). The T_g of a given amorphous phase is dependent on the T_g and mass percent of each component, including water (Angell, 1995; Franks et al., 1991; Levine and Slade, 1988, 1992; Pikal, 1994, 1999). Compared to excipients, dried proteins have relatively high T_g's (e.g., >150°C; Angell, 1995). Thus, with all other factors being held constant, the formulation T_g varies directly with the mass fraction of protein. However, care must be taken that the mass fraction of protein is not so high that there are not adequate levels of stabilizing excipients to prevent protein unfolding during lyophilization (Cleland et al., 2001; and see below).

Fortunately, sucrose and trehalose, which are the preferred excipients for inhibiting lyophilization-induced protein unfolding (see below), also provide a glassy matrix with acceptably high T_g values. For example, with water contents of 1% the T_g for pure sucrose and trehalose are about 100 and 65°C, respectively (Crowe et al., 1998).

It has now been documented with several proteins, that simply storing the formulation at temperature below T_g alone does not assure optimal stability. A

Samsung Bioepis Exhibit No. 1002
Page 7792

native protein structure is also required. For example, proteins lyophilized in dextran alone are usually unfolded, but in a glassy matrix with a relatively high T_g (e.g., > 75° C). Yet they still degrade at relatively rapid rates compared to those for native protein molecules lyophilized with either sucrose or trehalose (Kriel-gaard et al., 1998a, 1999; Lueckel et al., 1998; Allison et al., 2000; Yoshioka et al., 2000). On pharmaceutical time scales of several months of storage many degradative reactions are not coupled to the glass transition of a formulation. This is because on these times scales there is still significant molecular mobility, even at temperatures well below (e.g., more than 30° C) the T_g (Hancock et al., 1995; Duddu et al., 1997; Pikal, 1999; Yoshioka et al., 1999).

The Water Content is Relatively Low

Because of its very low T_g (–135°C), water is a potent plasticizer for glasses; increasing water content in the dried formulation will greatly reduce T_g. For example, increasing the water content of pure sucrose from 1 to about 3–4% (g H_20/100 g dried powder) is sufficient to reduce the T_g to below room temperature (Crowe et al., 1998). It is critical to achieve a sufficiently low water level for a given formulation such that T_g exceeds the planned storage temperature. The lyophilization cycle dictates the initial water content (see reviews listed above). The most important parameter is the temperature for secondary drying, when the unfrozen water is desorbed (Pikal et al., 1990).

Water can also be transferred to the product from the vial stoppers during storage (Pikal and Shah, 1992; DeGrazio and Flynn, 1992; Hora and Wolfe, 1999). This effect can be dramatic. For example, let's consider a formulation containing 10 mg of dried protein, 40 mg of sucrose and initial water content of 1% by weight. The total amorphous fraction containing protein and sucrose has 0.50 mg of water. If 1.0 mg of water was transferred from the stopper to the product, the water content of this fraction would increase from 1% to 3.0%. This increase would be sufficient to lower the formulation T_g to below room temperature (Crowe et al., 1998). The risk of transfer of moisture from stoppers can be minimized by drying the stoppers before use, and, if acceptable for a given product, using stoppers coated with a material such as Teflon (see Hora and Wolfe, 1999).

A Strong, Elegant Cake Structure is Obtained

Often the most desired cake has strong, porous structure, without macroscopic collapse or meltback. This structure has a high surface area to volume

Samsung Bioepis Exhibit No. 1002
Page 7793

ratio, which aids in the rapid dissolution of product upon addition of water. A detailed account of how to obtain such a cake structure is beyond the scope of the current chapter, but is available in several previous reviews (see above). For the current purposes it is sufficient to focus on the impact of formulation composition on avoiding collapse or meltback. When a product is frozen, the protein and amorphous excipients (e.g., sucrose) are dispersed between ice crystals and any excipient used as a crystalline bulking agent (e.g., glycine). To obtain an appropriate cake structure during lyophilization, the product temperature during primary drying, when the water in ice is sublimed, must be below the characteristic collapse and eutectic melting temperatures of amorphous and crystalline solutes, respectively. Above the eutectic temperature, the melting of crystalline solutes leads to massive loss of porous structure and macroscopic dissolution of the frozen matrix into a "puddle". Above the collapse temperature, which closely coincides with the glass transition temperature (T_g') of the maximally freeze-concentrated amorphous phase, the amorphous phase cannot support its own weight. The result is also a loss of pore structure and a macroscopic shrinkage or collapse of the cake.

It should be noted that the T_g' thermal event, which can be measured with differential scanning calorimetry, is also referred to as a softening event (T_s), rather than the actual glass transition temperature of the freeze-concentrated amorphous phase (Shalaev and Franks, 1995). Whatever the exact nature of the thermal event, it can be detected with differential scanning calorimetry as a second order increase in the baseline of the thermogram, which usually occurs just prior to the onset of the endotherm for the melting of ice (e.g., Her and Nail, 1994). The transition can also be measured with electrothermal methods (Her et al., 1994).

Another powerful method, which is essential for rational development of lyophilized formulations, for determining collapse and eutectic melting temperatures is freeze-drying microscopy (e.g., Nail et al., 1994). With this approach, the formulation of interest is directly examined visually for its performance during a simulated freezing, annealing and drying cycle. All of the critical phase changes, including ice formation, solute crystallization, eutectic melting and collapse, can be detected easily, and the temperature of their occurrence can be measured accurately.

Formulation composition dictates collapse temperature. Each pure amorphous excipient has a characteristic T_g' and collapse temperature; the collapse temperature for the formulation is the mass averaged temperatures of all of the components in the amorphous phase. It is important to design a formulation with maximum collapse temperature, because the rate of drying is directly proportional to the sample temperature during lyophilization. To allow for a reasonable drying time the T_g' should not be lower than –40° C. The T_g' values for pure sucrose is –32° C, while that of pure trehalose is –30° C (Skrabanja et al., 1994; Chang and

Samsung Bioepis Exhibit No. 1002
Page 7794

Randall, 1992). In contrast, glucose, which should also be avoided because it is a reducing sugar, has a T_g' of −43° C. The collapse temperature of pure protein is about −10° C, which means increasing the protein:sugar mass ratio will increase collapse temperature. Finally, collapse temperature will be decreased if salts and excipients are not maximally crystallized. For example, glycine has a T_g' of −37° C, and its contribution to the amorphous phase can reduce collapse temperature to impractically low values (Carpenter and Chang, 1996). This problem can be avoided by using the appropriate annealing (see Carpenter and Chang, 1996) of the frozen product to maximize crystallization.

Meltback can be avoided by using crystalline solutes with relatively high eutectic melting temperatures. For example, eutectic melting temperatures for mannitol and glycine are −1 and −4° C, respectively. In contrast, additives such as calcium chloride have very low eutectic melting temperatures (e.g., −51° C for calcium chloride). If mannitol or glycine is used as a crystalline bulking agent, the T_g' of the amorphous excipient phase, which is lower that the eutectic melting temperatures of these excipients, will be the value that dictates the temperature of primary drying.

Collapse can also occur during secondary drying, when unfrozen water is desorbed, if the temperature is increased too rapidly. As water is removed from the amorphous phase the T_g of this phase increases. Thus, product temperature can be raised gradually and collapse can be avoided if product temperature at a given time point does not exceed the T_g. Directions for optimizing secondary drying can be found in previous reviews mentioned above.

Steps Taken to Minimize Specific Routes of Protein Chemical Degradation

It is essential that the major routes of chemical, as well as physical, degradation be characterized carefully for each protein, because all of the criteria listed above can be met and a protein might still be damaged during long term storage in the dried solid. For example, methionine oxidation is a common degradation pathway for therapeutic proteins (Manning et al., 1989; Ahern and Manning, 1992; Cleland et al., 1993; Goolcharran et al., 2000). Even in a formulation that prevents protein unfolding and has a T_g exceeding the storage temperature methionine oxidation can proceed at an unacceptably rapid rate. If a methionine residue is on the surface of the protein, maintaining native structure would not prevent this residue's exposure to reactive oxygen species in the dried solid. But why would the glassy matrix not sufficiently retard the mobility of the reactive species to prevent the reaction from occurring? On pharmaceutical time scales (i.e., of many months) relevant motion in a glass is not arrested unless the storage

Samsung Bioepis Exhibit No. 1002
Page 7795

temperature is about 50° C below the T_g (e.g., Hancock et al., 1995; Pikal, 1994; Pikal, 1999). Therefore, the mobility of a relatively small reactive oxygen species and the oxidation of methionine residues are probably not coupled to the glass transition of the formulation. An intriguing alternative, but not mutually exclusive, explanation has been suggested by Steve Prestrelski (personal communication). He proposes that reactive oxygen species accumulating during long-term storage might not be causing damage just in the dried solid. Rather upon rehydration oxygen radicals rapidly react with the protein. This is an important area for future research.

Fortunately, despite the rather complicated and poorly understood theoretical aspects of unacceptable methionine oxidation rates in dried protein formulations, from a practical viewpoint dealing with the problem is relatively straightforward. Methionine oxidation can be minimized by sealing vials under nitrogen and/or using formulation additives (e.g., free methionine) to compete with protein residues for reactive oxygen species. These approaches should be effective whether the oxidation of residues occurs in the dried solid, during rehydration or at both times.

RATIONAL DESIGN OF STABLE LYOPHILIZED FORMULATIONS

Two critical practical issues need to be considered when choosing excipients for a therapeutic protein formulation. 1) From a financial standpoint, scientists should focus on using excipients and processing approaches that do not need to be licensed from a patent holder. Acceptable protein stability can be achieved readily using excipients and processing methods that are well known to someone, to use the legal term, "skilled in the art of protein formulation." Clearly, however, the specific applications of such compounds and processes to a given protein drug product and/or a class of proteins often can be patented. Such patents may be critical to the company developing that product.

2) From a regulatory standpoint (and financial perspective, also), the formulation scientist should choose from among excipients that are already used in approved parenteral products. For protein stabilizers, the best choices are the disaccharides, sucrose and trehalose. For bulking agents, the best choices are glycine, mannitol and hydroxyethyl starch. For surfactants, usually the Tweens (20, 40 or 80) are preferred.

Based on these practical concerns and the criteria for a successful lyophilized protein product (Table 1), prototypic rational formulations are presented in Table 2. If the formulations suggested are employed, and the criteria in Table 1 are met, then most likely the product will have acceptable stability.

Samsung Bioepis Exhibit No. 1002
Page 7796

temperature is about 50° C below the T_g (e.g., Hancock et al., 1995; Pikal, 1994; Pikal, 1999). Therefore, the mobility of a relatively small reactive oxygen species and the oxidation of methionine residues are probably not coupled to the glass transition of the formulation. An intriguing alternative, but not mutually exclusive, explanation has been suggested by Steve Prestrelski (personal communication). He proposes that reactive oxygen species accumulating during long-term storage might not be causing damage just in the dried solid. Rather upon rehydration oxygen radicals rapidly react with the protein. This is an important area for future research.

Fortunately, despite the rather complicated and poorly understood theoretical aspects of unacceptable methionine oxidation rates in dried protein formulations, from a practical viewpoint dealing with the problem is relatively straightforward. Methionine oxidation can be minimized by sealing vials under nitrogen and/or using formulation additives (e.g., free methionine) to compete with protein residues for reactive oxygen species. These approaches should be effective whether the oxidation of residues occurs in the dried solid, during rehydration or at both times.

RATIONAL DESIGN OF STABLE LYOPHIILIZED FORMULATIONS

Two critical practical issues need to be considered when choosing excipients for a therapeutic protein formulation. 1) From a financial standpoint, scientists should focus on using excipients and processing approaches that do not need to be licensed from a patent holder. Acceptable protein stability can be achieved readily using excipients and processing methods that are well known to someone, to use the legal term, "skilled in the art of protein formulation." Clearly, however, the specific applications of such compounds and processes to a given protein drug product and/or a class of proteins often can be patented. Such patents may be critical to the company developing that product.

2) From a regulatory standpoint (and financial perspective, also), the formulation scientist should choose from among excipients that are already used in approved parenteral products. For protein stabilizers, the best choices are the disaccharides, sucrose and trehalose. For bulking agents, the best choices are glycine, mannitol and hydroxyethyl starch. For surfactants, usually the Tweens (20, 40 or 80) are preferred.

Based on these practical concerns and the criteria for a successful lyophilized protein product (Table 1), prototypic rational formulations are presented in Table 2. If the formulations suggested are employed, and the criteria in Table 1 are met, then most likely the product will have acceptable stability.

Samsung Bioepis Exhibit No. 1002
Page 7797

Table 2.
Example of Rational Lyophilized Protein Formulation

1. Buffer that does not acidify during freezing (e.g., Tris, histidine, citrate)
2. Specific ligands/pH that optimizes thermodynamic stability of protein
3. Trehalose or sucrose to inhibit protein unfolding and provide glassy matrix
4. Bulking agent (e.g., mannitol, glycine or hydroxyethyl starch)
5. Nonionic surfactant to reduce protein aggregation

It is important to emphasize that not all components listed in Table 2 may be needed for a given protein product. For example, it may not be necessary to include a nonionic surfactant to aid in reducing protein aggregation. If there is not clear evidence that a given component is beneficial for a formulation, then that component should not be included in the formulation. Also, some components that are used because of "tradition" and/or for purposes such as final formulation tonicity can cause great difficulties in lyophilization. The most common example is the use of NaCl as a tonicity modifier. During lyophilization, NaCl can greatly reduce the collapse temperature of a formulation, if a fraction of the salt does not crystallize (Her et al., 1994). Crystallization of NaCl during freezing and annealing can be inhibited by other excipients (e.g., bulking agents and stabilizing sugars). Because of the low collapse temperature, a low temperature cycle must be used, which increases production time and costs. Also, even if formulations with NaCl can be lyophilized successfully in small research lyophilizers, there is great risk that a large fraction of vials will collapse during large scale manufacturing runs. Thus, if at all possible, NaCl should not be used in lyophilized formulations. Alternative tonicity modifiers include mannitol and glycine, which can also serve as crystalline bulking agents.

Every protein and product has unique characteristics, some of which may cause difficulty in designing stable formulations. Thus, the suggestions in Table 1 should be considered only as a good starting point in formulation development. For the remainder of this chapter we will discuss the rationale for the choice of each of the formulation components, their mechanisms of action and other practical approaches that can be used to increase protein stability.

Choice of Buffer

In terms of protein stability, the main concern with choice of buffer is the potential of certain buffer salts to precipitate during freezing and cause large changes in pH. For example, crystallization of the dibasic form of sodium

Samsung Bioepis Exhibit No. 1002
Page 7798

phosphate results can result in a pH < 4 (van den Burg, 1959; van den Burg and Rose, 1959; Anchordoquy and Carpenter, 1996). Thus, whenever possible, sodium phosphate buffer should be avoided. Although somewhat obvious, it is important to realize that a sodium phosphate system will be present if one starts with potassium phosphate buffer salts and NaCl, as is the case with phosphate buffered saline. Alternative buffers that do not appear to have major pH changes during freezing include Tris and histidine (Anchordoquy and Carpenter, 1996).

A clear example of the detrimental effects of buffer acidification on the stability of a lyophilized protein formulation has been reported by Lam et al. (1996). These researchers documented that a succinate buffer with an initial pH of 5 exhibited a pH drop of 1–2 units during lyophilization. The result was a reduced stability of interferon-γ during storage of the lyophilized formulation, relative to that noted for formulations prepared with glycolate buffer, which does not undergo freezing-induced acidification.

Specific Ligands/pH that Optimizes Thermodynamic Stability of Protein

Often experience gained during purification, intermediate storage protocols and preformulation studies can provide great insight into specific solution conditions (e.g., pH, ionic strength, buffer type) that confer the greatest stability to a given protein. Before choosing the appropriate "general" stabilizers, which are effective at protecting most proteins, it is absolutely essential that the formulation be optimized for the specific factors that increase the physical and chemical stability of a given protein. For example, simply avoiding extremes in pH can drastically reduce the rate of deamidation (Manning et al., 1989; Goolcharran et al., 2000). Also, specific ligands that increase protein physical stability (e.g., by increasing the free energy of unfolding) should be investigated. The stabilizing effects of heparin and other polyanions on growth factors (e.g., Chen et al., 1994; Volkin and Middaugh, 1996) and calcium on DNase (Chen et al., 1999) provide good examples. As will be described below, increasing the free energy of unfolding directly correlates with increased resistance of proteins to denaturation during freezing. In addition, at least in the case of DNase, there was also dramatic increases in storage stability of the lyophilized protein in formulation containing calcium, which were not due to increased structural stabilization during lyophilization (Chen et al., 1999). The mechanism for this effect is unknown.

Moreover, sometimes protein unfolding during freeze-drying can be minimized by optimizing initial solution pH (e.g., Prestrelski et al., 1995). However, in other cases it has been found that initial solution pH does not impact the degree of unfolding arising during lyophilization (Costantino et al., 1995; Carrasquillo et al., 2000). For each protein it is necessary to investigate the effect of initial pH

Samsung Bioepis Exhibit No. 1002
Page 7799

(and potential pH changes during freezing) on structural retention during lyophilization. Infrared spectroscopy can be used to monitor secondary structure in the dried solid, and the resulting data can be used to choose the initial pH that results in the most native-like secondary structure in the dried solid.

Trehalose or Sucrose to Inhibit Protein Unfolding and Provide Glassy Matrix

In this section we will discuss the rationale for choice of stabilizing excipients. Then we will describe the mechanisms for protection of proteins by additives during freezing and drying. Mechanistic insight is important for a clear understanding of protein stabilization during lyophilization and for guiding critical practical choices, such as determining the level of disaccharide needed for optimal protein protection. Finally, we will address this and other practical issues in the use of stabilizing excipients to inhibit protein unfolding during freezing and drying.

Rationale for Choice of Stabilizing Sugar. Among the numerous compounds tested, it appears that the most effective stabilizers of proteins during lyophilization are disaccharides (reviewed in Carpenter et al., 1999). Trehalose and sucrose are the best choices for a stabilizing disaccharide for therapeutic proteins. Both sugars: 1) protect proteins during both freezing and dehydration; 2) are nonreducing; 3) tend to remain amorphous during lyophilization; and 4) have been used in approved parenteral therapeutic products. There are, however, some important differences in the physicochemical properties of these sugars. 1) Trehalose has a higher T_g at a given moisture content than sucrose and, thus, for formulations containing trehalose it may be easier to obtain an appropriate cake structure with an economical lyophilization cycle (Crowe et al., 1998). However, a skilled process engineer should be able to design economical, effective cycles for formulations containing either sugar. In addition, the condition of having a T_g greater than the product storage temperature will hold at higher residual water contents for trehalose. In products with a relatively high protein concentration, the protein could contribute to an increased T_g, which serves to minimize the advantages of trehalose. 2) Trehalose is more resistant than sucrose to acid hydrolysis. Hydrolysis of these disaccharides produces reducing sugars, which must be avoided. Usually this is not a problem, unless very low pH's (ca. < 4) are employed. It should be noted that acid catalyzed hydrolysis of sucrose can occur even in lyophilized solids (Shalaev et al., 2000), suggesting that buffer acidification during freezing (see below) could ultimately result in formation of reducing sugars and resulting damage to proteins in a lyophilized formulation. 3)

Samsung Bioepis Exhibit No. 1002
Page 7800

Sucrose appears to be somewhat more effective at inhibiting unfolding during lyophilization (Allison et al., 1999). This difference has been most obvious when there is a relatively high protein concentration and a need to employ a relatively high initial concentration of sugar. Evidence to date indicates that less effective stabilization by trehalose is due to the greater propensity of this sugar to phase separate from polymers (Isutzu et al., 1996) and proteins (S.D. Allison, T.W. Randolph, B.S. Chang and J.F. Carpenter, unpublished observation) during freezing and drying. Whether or not this is a problem with a given formulation cannot be predicted. Hence, the relative capacities of sucrose versus trehalose to inhibit lyophilization-induced unfolding of must be examined for each protein. The final choice of sugar for a given protein product should be based on a direct comparison of sucrose and trehalose as stabilizers during the lyophilization cycle and storage in the dried solid.

Reducing sugars, such as maltose or lactose, should not be used. These compounds may effectively inhibit protein unfolding during the lyophilization cycle, but during storage in the dried solid they can degrade proteins via the Maillard reaction between carbonyls of the sugar and free amino groups on the protein (Hageman, 1992; Li et al., 1996).

Mechanism for Freezing Protection. Many different compounds, such as sugars, polyols, certain amino acids, methylamines and salting-out salts, nonspecifically stabilize proteins during freezing or freeze-thawing ("cryoprotection"). The same compounds have also been shown in nonfrozen aqueous systems to increase protein thermodynamic stability (e.g., increase resistance to temperature- or chaotrope-induced unfolding). Protection of proteins by these compounds during freezing and freeze-thawing is due to the same universal thermodynamic mechanism that Timasheff and colleagues have defined for solute-induced protein stabilization in nonfrozen aqueous solution (reviewed in Carpenter and Crowe, 1988; Carpenter and Chang, 1996; Carpenter et al., 1999; Timasheff, 1998).

Usually relatively high concentrations (ca. > 0.3 M) of solute are needed to stabilize proteins because the interactions of the solute with the protein are relatively weak. Stabilizing solutes are excluded preferentially from the surface of the protein. This statement does not mean that no solute molecules bind to the surface of the protein, but rather that there is a lower concentration of solute in the immediate vicinity of the protein than in the bulk solution. The chemical potential of the protein is increased in the presence of a preferentially excluded solute, which is the basis for the effect of solute of protein thermodynamic stability. Considering a two-state model for protein folding ($N \leftarrow \rightarrow D$), the native state is favored thermodynamically (e.g., under physiological conditions) because it has a lower free energy than the denatured state. The key to increasing the thermodynamic stability of the native state is to increase the free energy barrier

Samsung Bioepis Exhibit No. 1002
Page 7801

between the native and denatured states. So how can increasing the chemical potential of the native state result in stabilization? The degree of preferential exclusion and concomitant increase in protein chemical potential correlates directly with the protein surface area. The denatured state has a much greater surface area than the native state. Thus, in the presence of a preferentially excluded solute, the magnitude for the increase in protein chemical potential will be much greater for the denatured than that for the native state; the native state will be stabilized.

This same mechanism applies to the inhibition of smaller scale structural expansions of the native state, which may be sufficient to promote irreversible protein aggregation, by compounds such as sucrose (e.g., Chang et al., 1996a; Kendrick et al., 1997, 1998; Kim et al., 2000). Many proteins are known to form non-native aggregates from species with conformations that are not greatly different from the most compact native state. One example is the molten globule that has perturbed tertiary structure, but native secondary structure. However, even expanded species within that native state ensemble can participate in intermolecular interactions leading to non-native protein aggregates (e.g., Chang et al., 1996a; Kendrick et al., 1997, 1998; Kim et al., 2000). Preferentially excluded solutes shift the equilibrium between protein species towards that with the lowest surface area, i.e., the most compact species in the native state ensemble. As a result aggregation is inhibited.

Timasheff's preferential interaction mechanism also explains the influence of solutes on the degree of assembly of multimeric proteins. Preferentially excluded solutes tend to induce polymerization and stabilize native oligomers since the formation of contact sites between constituent monomers serves to reduce the surface area of the protein exposed to the solvent. Polymerization reduces the thermodynamically unfavorable effect of preferential solute exclusion. In this case, the assembled protein is much more stable than the constituent monomer because of the direct solute effects, but also because of the increase in oligomer stability gained by intersubunit contacts (e.g., Neet and Timm, 1994). In one example of this effect during lyophilization, maintenance of the native tetramer during freezing has been shown to increase the resistance of lactate dehydrogenase to dissociation and inactivation during subsequent drying (Anchordoquy and Carpenter, 1996; Anchordoquy et al., 2001).

How do we know that the preferential exclusion mechanism is actually operative in the frozen state? It is not possible to measure directly the protein-solute interaction in the frozen state. However, the effects of solutes on protein chemical potential and the resulting protein stabilization can be inferred from a freezing study with hemoglobin, in which the protein was partitioned into separate polyethylene glycol-rich and dextran-rich solution phases (Heller et al., 1996). In a phase separated system, thermodynamic equilibrium requires that chemical potential of each component be equal in both phases. Hence, there should be

Samsung Bioepis Exhibit No. 1002
Page 7802

equivalent effects of the different solute conditions in the two phases on the increase in free energy of protein unfolding. In other words, the impact of preferentially excluded solutes on protein stability is equal in the two phases. As predicted from the preferential exclusion mechanism, the degree of structural protection of hemoglobin during freezing (as observed directly in the frozen state with infrared spectroscopy) was equivalent in both phases (Heller et al., 1996).

Mechanism for Inhibition of Dehydration-Induced Unfolding. The interaction of a protein's residues with water are intimately involved with the formation of the native, globular protein structure, and if the protein is dehydrated it will unfold. It has been documented by numerous studies that sucrose and trehalose prevent dehydration-induced unfolding by hydrogen bonding to the dried protein in place of the lost water (e.g., Carpenter and Crowe, 1989; Prestrelski et al., 1993a; Allison et al., 1999; Costantino et al., 1998; Wolkers et al., 1998; Tzannis and Prestrelski, 1999). This so called "water replacement mechanism" is supported by several different observations.

For example, with infrared spectroscopy, it has been found that the band at 1583 cm^{-1} in the spectrum for lysozyme, which is due to hydrogen bonding of water to carboxylate groups, is not present in the spectrum for the dried protein (Carpenter and Crowe, 1989; Remmele et al., 1997; Allison et al., 1999). When lysozyme is dried in the presence of trehalose or sucrose, the carboxylate band is retained in the dried sample, indicating that the sugar is hydrogen bonding in the place of water. Similar results have been obtained with α-lactalbumin and sucrose (Prestrelski et al., 1993a). The magnitude of the retention of the carboxylate band correlates directly with the level of trehalose or sucrose in the lyophilized formulation, as well as with the degree of inhibition of unfolding (Allison et al., 1999). These effects of sugars on proteins in the dried solid are not due to the presence of increased amounts of water in the formulations dried with sugars (Allison et al., 1999; Tzannis and Prestrelski, 1999). The level of water in formulations dried with the sugars is as low as that for the protein lyophilized from just buffer or water, and is so low that the hydration shell of the protein is essentially completely removed (Prestrelski et al., 1993a; Krielgaard et al., 1998a, 1999; Allison et al., 1999).

Furthermore, Farhat et al., (1998) used infrared spectroscopy to study dried gelatin-sugar mixtures and the hydration behaviors of the mixtures, and concluded that the sugars hydrogen bond to the dried protein. Costantino et al. (1997) studied water sorption of proteins lyophilized with sucrose or trehalose and found that the solid-state interactions between protein and sugar reduced the availability of water binding sites. Tzannis and Prestrelski (1999) found that water sorption behavior of spray-dried protein formulations containing stabilizing levels of sucrose indicated hydrogen bonding between dried protein and the sugar. However, if an excessively high sucrose:protein ratio was employed, due to

Samsung Bioepis Exhibit No. 1002
Page 7803

formation of protein- and sugar-rich phases, hydrogen bonding of sucrose to protein and resulting protein stabilization were reduced. Also, Suzuki et al. (1998; 1999) found that there was a high degree of stabilization of lactate dehydrogenase when sucrose remained amorphous and hydrogen bonded to the dried protein. Crystallization of sucrose abolished hydrogen bonding between the sugar and dried protein, as well as protein stabilization.

Further support for the water replacement mechanisms comes from two studies that compared the relative effectiveness of saccharides of increasing molecular weight for inhibiting lyophilization-induced unfolding. Tanaka and colleagues (1991) found that the capacity to protect catalase during freeze-drying was inversely related to saccharide size. They suggested that as the size of the saccharide increases, steric hindrance interferes with hydrogen bonding between the saccharide and the dried protein. In support of this contention, the carboxylate band is only minimally detectable in the infrared spectrum of lysozyme freeze-dried in the presence of glassy dextran and the protein is unfolded (Allison et al., 1999). With infrared spectroscopy, Prestrelski et al. (1995) found that as the molecular weight of a carbohydrate additive was increased the capacity to inhibit unfolding of interleukin-2 during lyophilization decreased, and the level of protein aggregation after rehydration increased. Also, it was clear that protection of the protein did not correlate directly with the formation of a glass (all samples were found to be amorphous) or with the glass transition temperature of the sample (the T_g increased as carbohydrate molecular weight increased). Rather, there was a negative correlation between stabilization and molecular weight, which is to be expected if protection during drying is due to the water replacement mechanism.

An alternative mechanism for stabilization of proteins during dehydration states that proteins are simply mechanically immobilized in a glassy, solid matrix during dehydration (e.g., Franks, 1991). The restriction of translational and relaxation processes is thought to inhibit protein unfolding, and spatial separation between protein molecules (i.e., "dilution" of protein molecules within the glassy matrix) is proposed to prevent aggregation. It is clear that protective additives must partition with the protein into the amorphous phase of the dried sample. If the compound crystallizes during lyophilization it does not inhibit protein unfolding (Carpenter et al., 1993; Izutsu et al., 1993; Krielgaard et al., 1999). Also, spatially separating protein molecules can help favor refolding over aggregation during rehydration (Allison et al., 1998, 2000). However, as evidenced by the failure of glassy dextran matrices to prevent unfolding (Prestrelski et al., 1995; Krielgaard et al., 1999; Allison et al., 1998, 1999, 2000), simply forming a glassy solid is not sufficient for inhibiting dehydration-induced unfolding.

Practical Considerations. The protein mass in a unit dose (or multidose) vial of therapeutic protein is dictated by the amount to be given to the patient.

Samsung Bioepis Exhibit No. 1002
Page 7804

With a lyophilized product, which can be reconstituted to a volume different from the prelyophilization volume, the initial protein concentration can be varied without changing the total protein mass. Changes in initial protein concentration can affect relative loss of protein due to interaction with vial surfaces (e.g., Page et al., 2000), protein stability during processing, the collapse temperature of the formulation, the T_g of the final dried formulation and the lyophilization process itself. Thus, protein concentration can be an important variable to investigate during formulation development.

Increasing initial protein concentration leads to apparent increased resistance to denaturation during freezing (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). This phenomenon can be demonstrated by determining the percentage protein aggregated after freeze-thawing, which varies inversely with protein concentration. Increasing protein concentration can directly reduce freezing-induced protein unfolding because one component of protein damage during freezing appears to involve protein denaturation during formation of the ice-water interface (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). Assuming that only a finite number of protein molecules can be unfolded per unit area at this interface (Krielgaard et al., 1998b), increasing the initial protein concentration will lead to a smaller percentage of damaged molecules. Of course, other factors such as direct cold denaturation and freeze concentration of salts may predominate over surface denaturation during freezing. For practical purposes, it is not necessary to sort out the relative contribution of the various stresses to freezing-induced damage. Rather, is important to include realative high protein concentrations during early formulation development in order to increase the "intrinsic" resistance of the protein to denaturation. The greater the intrinsic stability of the protein, the lower the concentration of excipient that will be needed.

Another advantage of employing a relatively high initial protein concentration is that increasing protein : disaccharide mass ratio increases both formulation collapse temperature and T_g' of the final dried product. In addition, the formulation volume will be reduced, which will reduce the duration and costs of lyophilization.

Of course, if the ratio of excipient to protein is not sufficiently high, there will not be adequate stabilization of the protein during freezing and dehydration. Freezing protection depends on the initial bulk concentration of the sugar, and if the given protein is freeze-labile sometimes concentrations exceeding 5% (wt/vol) are needed to maximize stabilization. However, often the stability of the protein is sufficient during freezing that the level of sugar needed for optimal protection during lyophilization is dictated by that required for inhibition of unfolding during dehydration. Protection during drying depends on the final mass ratio between the sugar and the protein (Pikal, 1994; Carpenter and Chang, 1996;

Samsung Bioepis Exhibit No. 1002
Page 7805

Cleland et al., 2001). Recently it has been demonstrated that the sugar:protein mass ratio predicts not only degree of native structural retention during lyophilization, but also the long-term storage stability of a lyophilized protein (Cleland et al., 2001). Generally, a weight ratio of sugar to protein of at least 1:1 is required for inhibiting lyophilization-induced unfolding, with optimal stability being reached at around 3–5:1. In practice, with the protein concentration held constant, a range of sugar concentrations can be tested during formulation screening to discern the optimal concentration needed for retention of native protein in the dried solid. In general, the optimal sugar concentration for stabilizing the protein during lyophilization will also provide storage stability, if the final dried powder has a T_g well above the storage temperature. Finally, it should be stressed that the minimal effective level of sugar should be employed, because of the effect of changing protein:excipient ratio on glass transition temperatures. Also, if excess levels of sugar are employed there is an increased risk of sugar crystallization if a product should happen to be held at temperatures near or above the formulation T_g (e.g., Krielgaard et al., 1999).

Bulking Agent (e.g., Mannitol, Glycine or Hydroxyethyl Starch)

If the product has a relatively low mass of protein per vial, often it will necessary to have a bulking agent in the formulation to prevent the protein from being lost from the vial during drying and to form the product cake. Mannitol and glycine are examples of bulking agents, which can also serve as tonicity modifiers, that usually crystallize to a substantial degree during lyophilization (Pikal, 1994; Carpenter and Chang, 1996). A disaccharide protein stabilizer will need to be used in combination with these bulking agents. One drawback of mannitol and glycine is that often an annealing step is required to assure maximum crystallization. If a fraction of the bulking agent remains amorphous during lyophilization, there may be problems with obtaining a suitable cake structure, as well as a risk of excipient crystallization during subsequent storage in the dried solid (e.g., Carpenter and Chang, 1996; Carpenter et al., 1997). An alternative, amophous bulking agent is the polymer hydroxyethyl starch, which is used clinically as a plasma volume expander. Its main advantages in lyophilization are that it has a high collapse temperature (ca. −10°C), it forms strong cakes and it has a relatively high T_g for a given moisture content (e.g., >200°C at 2% residual moisture). However, as is the case with dextran, the large polymer hydroxyethyl starch does not inhibit protein unfolding during dehydration, and may actually foster additional protein unfolding due to phase separation from the protein during freezing and drying. Again, stabilizing disaccharides must be used with hydroxyethyl starch to inhibit lyophilization-induced protein unfolding.

Samsung Bioepis Exhibit No. 1002
Page 7806

Nonionic Surfactant to Inhibit Aggregation

Surfactants are often included in protein formulations to minimize interfacial denaturation and aggregation, e.g., at vial surfaces, due to bubble entrainment during filling or because of agitation (Chang et al., 1996c; Krielgaard et al., 1998b; Page et al., 2000). For a lyophilized product, a surfactant can be beneficial to minimize protein aggregation during vial filling, freezing and rehydration. Generally, a surfactant will not inhibit protein unfolding during dehydration (Krielgaard et al., 1998a). The mechanisms by which surfactants inhibit protein aggregation will be described in a separate chapter in this volume. For the current discussion it is sufficient to stress that a surfactant should not be included in a lyophilized product, unless there is direct evidence that increases recovery of native protein in the rehydrated sample. Surfactants can decrease the free energy of unfolding of some proteins, which may cause the compound to actually foster aggregation. Also, they have been shown to inhibit the assembly of small soluble aggregates into higher order soluble oligomers and insoluble aggregates (e.g., Krielgaard et al., 1998b). This effect can manifest itself during lyophilization and rehydration. As a result, the presence of a surfactant can cause an undesirable increase in the level of soluble aggregates.

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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
Page 7813

8

High Throughput Formulation: Strategies for Rapid Development of Stable Protein Products

Rajiv Nayar and Mark C. Manning

INTRODUCTION

With the growing demand for new and innovative medicines, drug companies are spending record amounts of money on research and development. In the U.S. alone, research and development (R&D) investments are expected to exceed $24 billion in 1999, with investments in biotechnology totaling about $7 billion (Pharmaceutical Research and Manufacturers of America, 1999). As a result, new technologies are increasing the efficiency of the drug discovery process, and the drug pipelines have more products than ever in development. Nearly 350 biotechnology-related products are currently in clinical trials and over 50 are on the market (Pharmaceutical Research and Manufacturers of America, 1999). Pharmaceutical companies can now typically assess potential activities of up to 100,000 compounds a day using high throughput screening systems. In the biotechnology arena, recent advances in genomics, functional genomics, proteomics, bioinformatics and pharmacogenomics are facilitating the development of protein drug candidates at a much faster rate than was possible during the early years of the biotechnology industry. Couple these changes with the impending

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Samsung Bioepis Exhibit No. 1002
Page 7814

publication of the complete sequence of the human genome by 2001 and there is a potential for an additional 15,000 protein drugs from the predicted 150,000 potential genes in the human genome.

This new wave of protein drugs will include compounds that can function as growth factors, act as specific stimulators or suppressors of certain functions or exhibit activities that have not been observed before. Many experts anticipate a golden age of protein-based therapeutics in the twenty-first century. As various research enterprises gear up to search for these new candidates using high throughput cellular and molecular screening assays, there undoubtedly will be a need for a corresponding ability to formulate these drugs much more quickly than in the past. In addition, there will be increased use of novel delivery systems to administer these protein therapeutics efficiently, making stabilization even more challenging. In short, the pharmaceutical biotechnology industry must find new ways to speed up the drug development process, in order to keep pace with the drug discovery process. We think that the limiting factor for introduction of new biotechnology-derived products will not be finding new drug candidates, but efficient and rapid formulation of these compounds into acceptable dosage forms for delivery and testing in humans.

The potential of many new drug candidates flowing through the pipeline will require implementation of an entirely new approach towards product development. The particular challenge addressed here is the demand that will be placed upon formulation groups, where there will be increased expectations to develop stable formulations of:

more proteins,
in a shorter period of time,
with less material, and
with little, if any, increase in personnel.

Therefore, one must consider new strategies for meeting such challenges.

This chapter is intended to serve as a forum for discussing and evaluating what factors must be considered in trying to implement new approaches to formulating protein pharmaceuticals in a rapid, rational fashion. As a comprehensive strategy for developing stable formulations of protein pharmaceuticals is not currently available, we have assembled what we consider to be the key elements necessary to accomplish this critical task. This chapter should not be viewed as a finished product, but as a work-in-progress, encouraging pharmaceutical scientists to comment on the concept and to modify the approach to their particular situations.

Again, the overarching goal is to possess a unified strategy for accommodating many more proteins at one time, while meeting shortening timelines. It should be expected that there would be a limited supply of well-characterized material and a fixed personnel head count. We envision a scheme that we term "high throughput formulation" or HTF. It is based upon our current

Samsung Bioepis Exhibit No. 1002
Page 7815

understanding regarding stabilization of proteins, but it also identifies areas where technical improvements must be made, especially in the area of analytical methodology and software. If the HTF strategy is successful, it should be possible to match the output of stable formulations by development groups with potential leads by discovery groups.

OVERALL STRUCTURE OF THE HTF APPROACH

The HTF concept includes four key elements, which are briefly outlined in Table 1. The heart of the HTF strategy is a decision plan, where the formulation scientist must decide on (a) the specific excipients to be used as well as (b) the final dosage form. Central to the HTF scheme is the use of a limited set of additives as well as dosage forms. Especially for lyophilized or freeze-dried formulations, our understanding of the behavior is such that some standard formulations can be envisioned (see Chapter 5). Also, the choice of basic solution conditions will be based upon preformulation studies that examine the behavior of the protein as a function of pH, ionic strength, and buffer type (see Chapters 1 and 3).

The second key aspect is an increased role for software and information databases. The preformulation studies could require a significant number of independent experiments to be conducted. Therefore, experimental design software should be used to reduce the number of experimental trials and optimize the results through statistical evaluation. In addition, there are a number of databases and associated programs for assessing physical and chemical properties based upon primary sequence of the protein through a number of websites (e.g. SWISS-PROT). These can provide insight into protein structure and other critical characteristics (e.g., pI, hydrophobicity and hydroflexibility plots and overall charge), and even identify residues that might be sensitive to chemical decomposition. We believe such capabilities will expand over time, and will play an increasingly important role in setting a rational plan for preformulation studies. Finally, we anticipate that specific protein stability databases will emerge, allowing scientists to gather data on specific development issues, without searching all of the open literature. Because information on protein stability comes from a multitude of disciplines (e.g., biochemistry, biophysics, food science, engineering, pharmaceutics, surface science and polymer science), open searches often miss crucial publications and documents. The challenge is not unlike Internet search engines trying to find a very specific subset of information on an ever-increasing world wide web. As these resources become more available and refined, they will emerge as invaluable tools for development of pharmaceutically acceptable formulations.

Samsung Bioepis Exhibit No. 1002
Page 7816

Table 1.
Key Elements of the HTF Approach

1. Decision Plan to Finalize Choice of Excipients and Dosage Form
   - A. Choice of Dosage Form
     1. Frozen Solution
     2. Liquid Formulation
     3. Lyophilized Formulation
     4. Protein Suspension
     5. Specialized Drug Delivery System
   - B. Choice of Excipients
     1. Proper pH/Buffer Species
     2. Salt
     3. Stabilizer
     4. Bulking Agent (for lyophilized formulations)
     5. Surfactant
     6. Chelators
2. Use of Software and Databases to Assist in Formulation Development
   - A. Experimental Design Software
   - B. Software and Databases for Analysis of Structure/Function/Properties/Stability
   - C. Protein Stability Databases
3. Availability of Essential Analytical Methods
4. Design of Appropriate Stability Testing Protocols

The third aspect of HTF is assembling the correct analytical methods to assay the stability, both chemical and physical, of a wide range of proteins. The mechanisms by which proteins degrade have been widely reviewed (Manning et al., 1989; Pearlman and Nguyen, 1992; Cleland et al., 1993), so they will not be discussed here. However, given this backdrop, we will describe certain key methods that must be available in order to conduct proper HTF studies. All of the required instruments do not necessarily need to be available in-house, as many can be accessed through contract organizations and through academic collaborations. In addition, it should be noted that there exists a tremendous opportunity to modify existing analytical techniques for use with smaller sample sizes and for rapid, parallel sampling. One of the expected limitations will be lack of well-characterized material. Whereas access to a minimum of 200–500 mg of protein for formulation studies is desirable, the genomic approach will tax the ability of process scientists to provide adequate amounts of purified proteins for all compounds under development. Therefore, in some cases, the total amount of protein available may be an order of magnitude less than the optimum. This situation should serve as an incentive to identify new approaches for gathering stability data. We will discuss below possible new methodologies that would be useful in the HTF scheme, but have not yet been applied to formulation development.

Samsung Bioepis Exhibit No. 1002
Page 7817

Finally, one must have a clear plan for stability testing, along with minimum criteria for a "stable" formulation. Certainly, that definition may change as a product matures. Minimum stability for the initial clinical studies may be quite different than the expectations for the final formulation tested in Phase III. Presently, there are debates within companies on how robust a preliminary formulation development or purification process must be in order to demonstrate efficacy in humans. Some companies will accept any minimal formulation in order to initiate clinical trials. However, even these formulations would likely have to have at least six to twelve months of shelf life. Our goal in the HTF approach is to develop a final formulation as early as possible in the drug development and testing process. If this goal can be accomplished, then fewer resources will need to be diverted to redesign the formulation later.

ROLE OF AN ESTABLISHED DECISION TREE FOR FORMULATION DESIGN

The central feature in the HTF scheme is a rational decision plan for choosing both the formulation components and the final dosage form. This decision tree must be flexible enough to accommodate a wide variety of proteins, it must allow for both liquid and solid dosage forms, and it must provide a rationale at each decision point. This framework allows the pharmaceutical scientist to decide what types of formulations are worth considering, both in terms of stability and the demands and desires of the marketing and clinical groups.

Often the design of a formulation of a protein pharmaceutical is not well developed. Companies attempt to expedite formulation selection by neglecting critical issues in formulation and stability. It is not uncommon to find sub-optimal formulations such as phosphate-buffered saline, possibly with the addition of human serum albumin as a ubiquitous protein stabilizer. For a few proteins, this approach has been sufficient, even resulting in commercially successful products, such as erythropoietin and granulocyte colony stimulating factor. However, there is a distinct danger in assuming that such successes with minimal formulations are generally applicable. Moreover, the current regulatory environment, and concerns in the general public surrounding the use of human-derived products in pharmaceuticals, makes the use of human serum albumin increasingly more difficult or even not acceptable. Therefore, there is pressure for any company selling a product that contains albumin to develop an albumin-free formulation. The same pressures are forcing companies to move from animal-derived surfactants to those obtained from vegetable sources. In addition, there is a realization that albumin, being a protein, is not indefinitely stable, and should have an expiration date above and beyond that of the active ingredient. All of these issues affect the

Samsung Bioepis Exhibit No. 1002
Page 7818

list of possible excipients that can be employed in a protein pharmaceutical product.

Constraints on a Pharmaceutically Acceptable Protein Formulation

Before we discuss the choice of dosage forms and excipients in the context of maximizing protein stability, we need to recognize the myriad of constraints placed upon the formulation by the rest of the company and by regulatory bodies. The first constraint, given the global nature of the pharmaceutical industry, is to have excipients that are acceptable in the major markets. This includes avoiding animal-derived additives, as discussed above. It also means that the list of possible additives is effectively limited to those already found in approved products in North America, Europe, and Japan. While this is not an immutable rule, few companies are willing to bear the added cost of getting a new excipient on the market while seeking approval for a new drug product. A major impact of this constraint is to move away from formulations containing albumin, which is still found in a number of protein products. One can anticipate that these older formulations will disappear in the coming years and be reformulated into second-generation products. A recent example has been the development of an albumin-free formulations for recombinant Factor VIII.

The second consideration is the tonicity of the final formulation. If a protein drug is to be administered by intravenous bolus injection or subcutaneously, rather than by continuous infusion, there are strict isotonicity and pH considerations that have to be met for a pain-free injection. Similarly, there are constraints on the choice of excipients, as some (e.g., citrate) have been shown to be irritating upon injection. This is one example of restrictions being set by the clinical groups, which should be clearly defined prior to the start of the formulation process.

Likewise, the eventual route of administration must be taken into account. A convenient administration system is highly desirable for ease of use in the clinical setting, so it is both a marketing and clinical issue. For example, in the case of a subcutaneous injection, there is a maximal volume (~1 ml.) that can be given to a patient without discomfort. Given the expected dose range, this will define the protein concentration that must be used. For other routes of administration, the ability to manipulate the protein concentration as a variable may be possible. Otherwise, it will be fixed, and the decision point regarding protein concentration is removed from the process described below.

The fourth issue is to work with a well-characterized bulk drug substance. This criterion will require cooperation and collaboration with the process science groups involved in fermentation and purification. Variation in the quality of the

Samsung Bioepis Exhibit No. 1002
Page 7819

starting material will compromise the validity of any preformulation or stability work done to date, as well as potentially invalidate data from toxicological tests.

The fifth constraint to be determined is the minimal stability required to complete proof-of-principle investigations, meaning animal studies and initial clinical trials. These investigations can extend for up to one year. Hence, at least a one-year shelf life for even the initial formulation may be required.

Finally, the manufacturing of the protein product must be possible at reasonable costs and allow adaptation to standard manufacturing processes. It is imperative that manufacturing economic analysis be conducted as soon as possible in the drug development process, including an assessment of whether sufficient manufacturing capability exists if the product is successful. Novel formulations that require special manufacturing setups and processes may not offer the advantages of speed which is necessary when one is confronted with development of numerous protein drug candidates. Most likely, specialized dosage forms and/or drug delivery systems will only be investigated with partners who possess the technology and capacity to solve these critical manufacturing issues. In addition, as the in-house pilot plants are challenged with manufacturing multiple products, with limited facilities and resources, there undoubtedly will be pressures to outsource the first clinical manufacturing because of both cost-effectiveness and timescale issues.

Proper Choice of Dosage Form

Considering all the therapeutic protein products on the market, it appears that three dosage forms are viable. The most preferred would be a solution formulation that is typically stored in the refrigerator and preferably in a pre-filled syringe. Second, a frozen solution loaded in a syringe or vial and thawed at the site of administration. Third, a lyophilized formulation, which can be stored at room temperature and reconstituted when needed. Each one of these dosage forms offers various advantages and disadvantages, in the speed of development, manufacturing, packaging and shipment logistics, and in administration of the product to the patient.

Generally, a frozen protein formulation offers the advantages of rapid development provided the protein is stable after multiple freeze/thaw cycles. It is also important to note that freezing can lead to selective precipitation of certain buffer species, producing possibly large pH shifts (van den Berg, 1959; van den Berg and Rose, 1959). This variation in pH, and the potential for damage at the water-ice interface (Chang et al., 1996; Hsu et al., 1995; Krielgaard et al., 1998), might limit the use of such a dosage form. Furthermore, there are disadvantages associated

Samsung Bioepis Exhibit No. 1002
Page 7820

with a frozen formulation concerning the packaging complexities involved in labeling, storage and maintaining the cold chain during transportation of a frozen product. These factors can add significantly to the development costs.

The second option for dosage form is a stable liquid formulation that can be stored in the refrigerator or at room temperature. This dosage form offers the advantages of potentially rapid development and ease of administration. However, for most proteins maintaining physical and chemical stabilities in aqueous solution for an extended period of time is extremely difficult. In addition, because the protein is free in solution there is potential for adsorption to the vial, whether it is made of glass or some type of plastic. A liquid product will have to be shown to be impervious to damage at the air-water interface during transportation, because agitation-induced denaturation and aggregation is a common problem with therapeutic proteins. There is also the possibility of leaching of metals and organics from the container and stopper, which is why some of the vials will need to be inverted during stability testing. These problems have been reported for drug products of all types (Airaudo et al., 1990; Nedich, 1983). Leached materials are particularly problematic with plastic vials and non-Teflon-coated stoppers. With glass vials the leachables could include ionic species that alter the pH and ionic strength of the solution during storage. Finally, the integrity of the container closure must be demonstrated, in order to ensure sterility of the product. For multi-use vials, preservatives can be added, but some are known to foster degradation of proteins (Lam et al., 1997; Rodrigues-Silva et al., 1999). Therefore, development of a stable liquid protein formulation has to be considered very carefully. Determination of the primary pathways responsible for decomposition of the active therapeutic protein is critical for developing this particular dosage form. All of these issues can add significant costs in terms of resources and analytical testing.

Most protein pharmaceuticals currently on the market are sold as lyophilized formulations. Placing proteins in the dried state significantly increases the shelf life, if the proper formulation and lyophilization cycle are used. The combined stresses of drying and freezing are inherently damaging to proteins, and only proper formulation will ensure that a stable product will be obtained. Fortunately, enough is understood now about freezing and drying of proteins that rational design of lyophilized formulations can be undertaken. Moreover, there are some formulations that seem to work well for a variety of proteins. We suggest that part of the strategy for rapidly identifying viable formulations is to make liberal use of some standard protein formulations. The rationale for these compositions has been described in detail in a number of review articles and book chapters, and is discussed in Chapter 5 (MacKenzie, 1976; Franks, 1990; Pikal, 1985, 1994; Carpenter and Chang, 1996; Carpenter et al., 1997). An approach towards developing a lyophilized formulation is described below.

Samsung Bioepis Exhibit No. 1002
Page 7821

As other formulation possibilities become more developed, one can modify the decision tree to accommodate them. For example, one can imagine alternatives to lyophilization for obtaining solid dosage forms. Both spray-drying and spray-freeze drying are being studied. However, it is expected that the formulation strategy for these processes will be quite similar to that used for lyophilization.

The most likely future additions to the dosage form list will be suspensions and specialized drug delivery systems. Protein suspensions can be produced by a number of different methods. The protein could be dried and suspended in a non-aqueous medium. Alternatively, the protein could be salted-out, using preferentially excluded solutes, such as ammonium sulfate or sucrose, leading to an aqueous vehicle containing most of the protein in its native state but in a solid form, because the solubility limit has been exceeded. The same effect might be accomplished by adjusting the pH to be near the isoelectric point of the protein. For the current version of HTF, we have chosen not to include these dosage forms until their use becomes more widespread and manufacturing facilities are developed to accommodate production of clinical batches.

The same can be said of specialized drug delivery systems, such as polymeric microspheres. Poly lactide/poly glycolide microspheres for sustained delivery of peptides have been in use for years. Recently, reports of their use for proteins, such as human growth hormone, have appeared. These systems bring their own specific stability issues (Cleland, 1998) in addition to making analysis of the stability of the active ingredient more challenging (Yang et al., 1999).

As discussed above, input from various departments, such as discovery research, pharmacokinetics, toxicology, purification, manufacturing, clinical and marketing, must be considered when deciding on the most appropriate dosage form for a given product. In the past, the drug development process has been more or less sequential, going from research to development to clinical, to manufacturing, and then to marketing. A change in strategy is required where there is more interactive, simultaneous communication between the various drug development departments. With the proper information from the other units, one should rapidly be able to decide on dosage form and develop candidate formulations.

Preformulation Studies

The exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation. Therefore, determination of the relative importance of various degradation pathways and elucidation of instability mechanisms for a given protein are essential. Even for closely related proteins, the relative stability and major

Samsung Bioepis Exhibit No. 1002
Page 7822

pathways for degradation might be quite different. Thus, the preformulation studies of new protein entities become very important and crucial tasks in the overall HTF strategy.

Preformulation refers to experimental studies designed at understanding the chemical and physical behavior of the protein or drug candidate. The major focus is on the solubility and stability as a function of a number of extrinsic factors, such as pH, protein concentration, ionic strength, buffer composition and temperature. This information will guide the appropriate choice of excipients, buffers and final protein concentration. Although a number of studies could be envisioned, HTF requires that these studies be very specific and focused. Any information that may narrow the choices would be essential for speedy formulation development. Relevant data can be gathered from the process-engineering group responsible for purification or literature studies on similar proteins, even if it is anecdotal. The final dosage form must also include constraints from marketing, clinical and regulatory groups addressing issues related to eventual approval of a pharmaceutically acceptable formulation for market. Ultimately, all of these data may be required to ascertain the most beneficial composition of excipients and the most appropriate dosage form for the protein product in hand.

Typical time scales for preformulation can range from one to three months depending on what assay systems have been established by the research groups and whether they can be utilized as stability-indicating. Therefore, early involvement of all of the formulation experts is critical for rapid development of lead formulations during the developmental phase. An additional driving force is also the fixed time required to generate the stability data to validate the formulation choice.

Proper Choice of Excipients

Liquid Formulations. If a solution dosage form is indicated, then there will be a finite set of possible excipients, restricting choices to those that are found in approved products and have been shown to be effective in protein formulations. For solution formulations, a list of possible excipients is given in Table 2.

Given that most protein formulations will exist at pH values between 4 and 9, there is a limited set of buffers that will exhibit sufficient buffering capacity. A number have been used in protein formulations and they are listed in Table 2. The main criteria for selection are good buffering capacity at the desired pH and lack of ability to accelerate specific chemical and physical reactions. Some proteins can preferentially bind certain buffer species, altering the conformational stability of the protein. In addition, some buffers (e.g., phosphate, Tris) have been

Samsung Bioepis Exhibit No. 1002
Page 7823

Table 2.
Possible Excipients for Use in Liquid Formulations

Excipient Class	Choices
Buffers	Histidine, Succinate, Acetate, Citrate Phosphate, Tris, Carbonate
Salts	Sodium Chloride, Calcium Chloride Magnesium Chloride
Non-Specific Stabilizers	Sucrose, Trehalose, other sugars, Amino acids (e.g., lysine, glycine)
Specific Stabilizers	depends upon the protein
Surfactants	Tween 20, Tween 80, Pluronic F-68 Sodium Dodecyl Sulfate
Chelators	EDTA

reported to catalyze certain hydrolytic reactions, such as deamidation (Patel et al., 1990).

Because proteins are polyelectrolytes, the ionic strength of the medium can often have an effect on structural stability. The most common salt used in protein formulations is sodium chloride. For an isotonic product, often it is advantageous to keep the concentration of salt as low as possible. Otherwise, the ability to employ stabilizing excipients will be limited.

Structural stabilization of proteins by specific ligands or cofactors in solution formulations can be important for formulation optimization. The mechanism by which these compounds increase thermodynamic stability is described by Timasheff (1992, 1995, 1998) and in other Chapters in this book. Several pharmaceutically important proteins have been stabilized by addition of specific ligands (including metals ions such as calcium), including fibroblast growth factor (Volkin et al., 1993) and DNAse (Chen et al., 1999).

To stabilize proteins (both in aqueous solution and during freezing) with non-specific compounds (e.g., sugars), relatively high concentrations (ca. > 0.2 M) of ligand (solute) are needed to affect protein stability. The mechanism of such stabilization is described in more detail in Chapters 2 and 3. The main point for the current discussion is that in the presence of nonspecific stabilizing excipients fewer protein molecules are unfolded at any point in time, either partially or completely, which reduces the amount of aggregation-competent species and the rate of aggregation (Kendrick et al., 1997, 1998). Addition of small amounts of a specific ligand should theoretically accomplish the same outcome. The most effective non-specific stabilizers tend to be disaccharides, such as sucrose and trehalose. However, certain salts, amino acids, and polymers are preferentially excluded as well. Still, unless there is evidence for advantage in use of a

Samsung Bioepis Exhibit No. 1002
Page 7824

particular compound from this group, sucrose and trehalose should remain the first-line choices.

Finally, one must decide on whether to include a surfactant in the formulation. Surfactants have been shown to inhibit protein aggregation during agitation (e.g., Bam et al., 1995; Krielgaard et al., 1998). The mechanisms by which surfactants stabilize proteins are discussed in a separate Chapter. The surfactant often will be most effective at or above the critical micelle concentration. In Table 2, only the surfactants that have been approved for use in parenteral products in the U.S. have been listed. Note that one potential drawback of including surfactants in the formulation are contaminants (e.g., peroxides) that could cause chemical degradation of proteins (e.g., oxidation).

Lyophilized Formulations. It can be assumed that most proteins will not exhibit sufficient stability in aqueous solution to allow a liquid formulation to be developed. Our understanding of the basic requirements for obtaining a stable lyophilized protein formulation is relatively well developed. The question then is what combination of excipients will allow such a formulation to be prepared. The minimal composition includes a buffer species, an additive capable of forming an amorphous glassy state and inhibiting lyophilization-induced unfolding in which the protein remains entrapped, a bulking agent to provide cake stability, and possibly a surfactant to retard surface-induced damage and/or promote refolding. The rationales for the minimal criteria for a stable lyophilized formation and the choice of appropriate excipients are described in detail in another Chapter.

While a comprehensive formulation development algorithm is yet to be published, following the HTF process outlined above, one can imagine obtaining a rational approach towards selecting final candidate formulation that can be used routinely, even with new classes of therapeutic proteins. Clearly, the details of each case will be guided by the outcomes of the preformulation studies. However, even without a complete data set from the preformulation experiments, implementation of a few standard formulations should be possible. Ultimately, we believe there will be a relatively small number (six of less) of generic, standard lyophilized formulations that will work for nearly all proteins. Each of these will have a corresponding lyophilization cycle that can be used to achieve optimal results. When the HTF process has evolved to this optimal state, the personnel demands be greatly reduced (in terms of full-time equivalents, or FTEs) and the time lines should shrink significantly from the current average of nearly one year.

Estimates of Resources Needed for Formulation Development

The current estimates of the resources required and the possible timelines for formulation development are given in Table 3. Specialized formulations

Samsung Bioepis Exhibit No. 1002
Page 7825

Table 3.
Estimated Resources Needed for Development of Final Stable Formulations of Protein Pharmaceuticals

Resource	Current Status	Initial HTF Targets	Optimal HTF Targets
• Personnel			
Standard Formulations	2 FTE	2 FTE	2 FTE
Complex Formulations	3–5 FTE 3–4 FTE	3 FTE	
• Time Scale			
Standard Formulations	12 months	6–9 months	3–6 months
Complex Formulations	12–24 months	12 months	6–9 months
• Minimal Amount of Material			
Standard Formulations	200–500 mg	100–200 mg	50–100 mg
Complex Formulations	400–2000 mg	200–500 mg	100–200 mg

(suspensions and controlled release dosage forms) will require more resources and less aggressive time lines (see Table 3). However, even this situation should improve as more of these types of products enter the marketplace. We anticipate that a significant number of these types of products will be approved over the next ten years. By then, most large pharmaceutical companies will have acquired the necessary expertise to develop them at rates comparable to the standard formulations of today. Once the HTF scheme has evolved to this stage, a company should be able to develop final formulations quickly and easily, even if personnel levels are relatively constant.

Interestingly, while a streamlined approach towards final formulation selection is the heart of the HTF strategy, it is the one aspect that can be introduced into any current product development scheme almost immediately, as it can be accomplished solely based on the current state of understanding of protein stabilization. Other aspects of the HTF package will demand more time to implement, as they will require significant advances to be made, particularly in the areas of analytical methodology and information technology. These two features of the HTF scheme are described below.

USE OF SOFTWARE AND DATABASES TO ASSIST IN THE HTF PROCESS

Unless formulation scientists have access to all pertinent information, critical choices for formulation components could be delayed or, even worse, incorrect. Therefore, we propose that a key aspect of the HTF process is to develop an integrated information system that can assist the scientist in making critical

Samsung Bioepis Exhibit No. 1002
Page 7826

decisions. Each and every step in the formulation development process is predicated upon having the most recent and comprehensive information on the physicochemical properties of the protein under development, as well as on the effects of additives on protein structure and stability. Clearly, the latter part of this goal is ever evolving as our knowledge of protein chemistry increases. Therefore, the final HTF scheme will involve a central database of essential information. While a large amount of this type of information is currently available, it appears in a multitude of sources, generated by researchers in disparate disciplines, ranging from materials science to biochemistry to food science to pharmaceutics. This makes access to the data difficult. Unless efforts are made to assemble this data in a cohesive package soon, the challenge will become even greater. Once the task is accomplished, sufficient information should be available to predict the performance of certain types of formulations.

In the absence of such resources, one can still make use of existing software and databases. These are summarized below. Briefly, they cover three areas: predictive protein algorithms based upon primary sequence, sequence homology analysis software, and programs aimed at predicting relative stability of proteins. Summaries of the locations and availability of these programs and databases has recently been published (Cook, 1999; Apweiler, 1999).

Predictive algorithms allow one to analyze a primary sequence and predict a variety of chemical and physical properties. For example, there has been enormous effort to develop schemes whereby one can predict the three-dimensional folding of a protein based solely on its sequence. Although there have been some successes, it has been demonstrated in double-blind studies that analysis of new sequences, when they have no clearly related partner in the existing database, fail to provide an accurate prediction of the folded conformation (Orengo et al., 1999; Vencloyas et al., 1999). When the analysis can be guided by sequence homology, the accuracy rises dramatically. Likewise, one can imagine developing structure-stability relationships, based solely upon primary sequence. Some studies of this nature have been reported. For example, Pandit and co-workers relate the frequency of certain dipeptide sequences to instability in proteins (Reddy, 1996; Guruprasad et al., 1990). The result is an algorithm that predicts the relative stability of a protein. Although the degradation criteria are based upon intracellular stability, this approach does provide researchers with an expectation of the overall fragility of the protein in question.

In another example of this type of sequence analysis, Roger et al. (1986) identified regions in proteins that were high in proline, acidic side chains (Asp and Glu), serine, and threonine. The presence of these regions was associated with more rapid degradation *in vivo*. Similar correlations should exist for *in vitro* stability. Even programs to predict likely deamidation sites (where Asn is converted into Asp-like residues) would be helpful, especially as our ability to remove those sites by recombinant DNA technology improves. There is now a

Samsung Bioepis Exhibit No. 1002
Page 7827

solid basis on which to predict both primary and secondary structure effects on deamidation rates (Wright, 1991; Xie and Schowen, 1999), which is considered in more detail in Chapter 4.

Given that numerous protein sequences, whether determined directly or extrapolated from nucleotide data, are available in databases that can be accessed via the world wide web, focused studies generating data on specific instabilities (e.g., deamidation, oxidation, aggregation) could lead to similar structure-stability relationships. The number of available sequences has been doubling every 18–24 months over the last ten years (Cook, 1999). Similarly, there has been an explosion in algorithms and software to analyze these data. Many focus on sequence homology and identification of functional and genetic relationships. Given these advances, it is not unreasonable to expect that stability-indicating profiles could be developed as well.

In addition to stability predictions, many programs are available to calculate important chemical and physical properties such as the isoelectric point, the hydrophobicity profile, the positioning of secondary structure elements, and the conformational flexibility. Knowledge of these properties would help the formulation scientist anticipate the behavior of a new molecule provided for development into a potential drug product. Furthermore, algorithms to predict correlations of these physical properties with physical and chemical stabilities of therapeutic proteins should be straightforward to design and implement with current programs.

ESSENTIAL ANALYTICAL METHODS

One must have access to certain equipment in order to characterize properly protein degradation pathways and effects of formulation variables on protein stability. In addition, we will discuss some areas where technological advancements must be made in order to allow formulation scientists to work with smaller amounts of protein. While a number of reliable and accurate stability-indicating assays exist, there needs to be efforts to miniaturize some of them. This will allow development of proteins where the amount of material is limited, e.g., less than 100 milligrams total.

The first analytical methods that are needed are those that can quantify the protein concentration. As determination of accurate extinction coefficients is straightforward, ultraviolet absorption spectroscopy is inexpensive and preferred over chemical methods, such as the Lowry method. One also needs to be able to monitor the global structure of the protein. Circular dichroism (CD) spectroscopy allows one to follow changes in both secondary and tertiary structure of proteins in solution (Manning, 1994). Infrared spectroscopy can also be used to detect

Samsung Bioepis Exhibit No. 1002
Page 7828

secondary structural changes (see below), and fluorescence and derivative U\ spectroscopies can be used to study tertiary structure.

The second category of required analytical methods are those that monitc retention of native primary structure, namely, chromatographic methods, suc as reversed phase HPLC and ion exchange HPLC, or alternative methods lik capillary electrophoresis. Among the most useful applications of these HPL(methods is the generation of a "tryptic map", which is accomplished by digest ing the protein with a sequence-specific enzyme (e.g., trypsin) and separating th resulting peptide fragments by reversed phase HPLC. The pattern then become a sensitive fingerprint of the protein. Any chemical modification of the protei will cause one or more of the peaks to shift, indicating damage to the protein Mass spectrometry can be used to determine the specific chemical modificatio leading to the peak shift.

Among the most important degradation pathways for proteins is aggrega tion. A number of methods are available to characterize and quantify aggregatio levels in proteins, including polyacrylamide gel electrophoresis (PAGE), siz exclusion chromatography (SEC), light scattering techniques and analytical ultra centrifugation. Although each of these methods has its advantages and disac vantages, all four should be considered essential, as they can be complementary They allow both qualitative and quantitative characterization of the soluble an insoluble aggregates in a bulk substance or final formulation.

For lyophilized formulations, a certain set of equipment is considered to b the minimum required for conducting formulation development properly. Thes include an infrared (IR) spectrometer, a Karl Fischer titrator, a differential scar ning calorimeter (DSC), and x-ray diffractometer. The IR instrument will allo\ one to determine the extent of structural damage (at a secondary structure level in the solid state. In addition, it can be used to monitor the secondary structur composition in the original solution (prior to lyophilization), in the frozen soli and in the reconstituted product (Carpenter et al., 1998). X-ray diffraction c solids allows one to determine the crystallinity of a solid sample. In formulation where the bulking agent is crystallizing during the lyophilization process, on needs to know whether the crystallization is complete. Incomplete crystallizatio can lead to degradation during storage (cf. Carpenter et al., 1997). The Ka Fischer apparatus allows one to measure the amount of water in the final cak Typically, moisture contents need to be at 1–2 % in order for the product to hav the optimal glass transition temperature (T_g) and maximal stability. Finally, DS(is required to measure the T_g of the final formulation. The storage temperatur must below the T_g. As the minimal acceptable storage temperature for lyophilize formulations is room temperature, a minimum T_g would be about 40° C. In add tion to these instruments, access to a microscope equipped with a lyophilizatio stage is valuable.

The issue of how to develop formulations with limited amounts of materi is a critical concern. One can imagine adapting a number of existin

Samsung Bioepis Exhibit No. 1002
Page 7829

technologies to assist in assessing the viability of a given formulation while using very small amounts of material. These would include:

1. microtiter plate assays,
2. robotics to assist in sample preparation and analysis,
3. ELISA or other antibody-based recognition assays for intact drug,
4. multi-sample adaptations of existing analytical techniques (e.g., DSC or analytical ultracentrifuge)
5. nanoseparation methodology (the so-called "lab-on-a-chip" approach),
6. surface-immobilized analytical methods, such as the BIAcore.

Each of these methods could provide significant increases in the capability to monitor the stability of a protein in a microformulation (less than 10 μg of active drug). For example, new chip technologies (either DNA- or protein-based) are becoming powerful tools that are increasingly used in all areas of drug discovery. They offer significant advantage over older technologies in terms of speed, sensitivity and cost. It is important to consider the use or implementation of these tools in formulation studies. One can envision protein chips doing for the study of proteins what DNA chips have done to the study of genes. That is, they can be used to identify critical parameters required for rapidly developing a stable formulation. Similarly, any of the other methods could be modified to assist the formulation scientist.

Finally, there has been a steady improvement in protein analysis tools such as mass spectrometry and more powerful and versatile biochemical techniques, which are not only more sensitive but offer the advantages of speed and low cost. So with the emerging technological advances in analytics and implementation of these enabling technologies in preformulation and formulation studies, one can envision a more time and resource efficient process for developing stable protein pharmaceuticals. Most of these technologies are being developed to meet the needs of efficient drug discovery research. The challenge here will be the speed at which the formulation scientists adapt these technologies into their programs. Unfortunately, these tools have not yet reached their maturity, so the implementation of micro-analytical methods for preformulation and formulation is crucial for developing HTF strategies for protein pharmaceuticals.

STABILITY PROTOCOLS

In addition to formal storage stability protocols that would be acceptable to regulatory agencies for real-time data, one must have standard approaches for assessing the possible long-term fate of the candidate formulations. Given the aggressive time line of the HTF strategy, one must rely, to some degree, upon accelerated storage studies to guide decision about suitable excipients. These

Samsung Bioepis Exhibit No. 1002
Page 7830

could be done as isothermal studies, storing the protein at room temperatu and 40° C, for example. In certain cases, exposure to higher temperature such as 50° C or even 60° C, may be warranted. However, it is important to no that at higher temperatures, there is the distinct possibility that the rat determining pathway may change compared to that at the projected stora conditions.

An alternative to isothermal testing that needs to be considered is the use non-iosothermal stress testing. In this procedure, the sample is exposed to a line increase in temperature over time (or approximately linear with respect to time Samples are taken at set intervals and assayed for protein damage. Using an alg rithm developed by Lee and Stavchansky (1998), it is possible to determine t Arrhenius parameters in a single experiment, which would otherwise require least three separate isothermal studies. This approach has been demonstrated f a pentapeptide (Lee and Stavchansky, 1998), and should be extended to larg proteins in the future.

UNIFIED STRATEGY FOR HTF

The requirements for HTF can be summarized in four points. First, integ tion of speed, sensitivity and cost can be achieved by having in place go analytical methodologies for monitoring both chemical and physical instabil mechanisms. These methods can serve as tools for elucidating the mechanism instability of protein drugs and also as stability indicating assays during analy of the final formulation. Second, identification of the instability mechanism proteins can lend itself to developing a fast-track stable liquid protein formu tion, although the disadvantages of addressing biocompatibility and transpor tion issues have to be addressed with such dosage forms. A third requirement HTF is to have obviously a safe and a convenient dosage form for administ tion. Coupled with this criterion should be the requirement for manufacturi the formulation using standard processes, so that if needed the production can out-sourced to a contract facility.

Finally, technological advances will be a major driver for HTF and in gene biotechnology development. The ability to work at low microgram to milligr scale of protein is essential for the ability to start the process before the d candidate achieves development status. In many cases, companies may initia neglect formulation and stability issues that will haunt them in later stages development. The development of successful formulations is dependent u the ability to study both the *in vitro* and *in vivo* characteristics of the prot drug, as well as its intended application in the clinic. Thus, it is essential the formulation group to work closely with the discovery research,

Samsung Bioepis Exhibit No. 1002
Page 7831

pharmacokinetics and the toxicology departments prior to the decision to move the drug into full-scale development. Resolving the difficulties in the early research and development stages will not only help in targeting the appropriate drug candidate for development, but also speed up the selection of a pharmaceutically acceptable formulation for clinical trials. What we propose is the involvement of the formulation group in a multi-disciplinary environment where interactions start early in the research environment. The appropriate lead candidates would be selected not only based on efficacy studies, but also on pre-formulation studies. We envision these studies to take one to three months to establish appropriate profiles for formulation development. As a result, the lead formulation can have the advantages of demonstrating efficacy and potential stability of the protein drug.

During the development process of a biotechnology drug we envision formulation to take typically three to six months. This period would encompass preformulation studies and evaluation of the formulation options determined by the planned clinical studies. Hence, protein formulation and purification can be developed concurrently using limited quantities of the product, and identification of the lead formulation could occur at the time of establishment of the purification process. What we propose is not a sequential development pathway for protein drugs, but a concurrent scenario where formulation design is initiated very early in the drug development pipeline. We are quite confident that the HTF can be coupled successfully with high throughput screening strategies of protein drugs and facilitate drug development within the inherent time and resource constraints of the pharmaceutical industry.

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Samsung Bioepis Exhibit No. 1002
Page 7834

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Samsung Bioepis Exhibit No. 1002
Page 7835

Index

Acid-soluble spore proteins (SASP), 97–98
Acidic fibroblast growth factor (aFGF), 69
Administration, route of, 182
Adsorption, 13, 152, 162–164
Aggregation
  covalent and non-covalent, 13
  nonionic surfactants to inhibit, 127
Agitation, and stability, 71–72
Analytical instruments, 3
Analytical methods, essential, 180, 191–193
Antibodies: *see* Recombinant humanized anti-IgE monoclonal antibody
ArgHCl, 45–46
Arrhenius equation, 8
Asparagine (Asn) residues, deamidation of in peptide and proteins, 86–92

Bax, 169
Bioactivity, 49
Bovine pancreatic trypsin inhibitor (BTPI), 40
Bovine serum albumin (BSA), 154–155
Bovine somatotropin (BSTI), 91
Büchi laboratory spray dryer, 139–141, 146, 147
Buffers/salts, 14, 67
  choice of, 118–119
  debye screening, 67–68
  ionization enthalpy, 68
  lysis, 33
  preferential exclusion of salts, 67
Bulking agent, 14, 126
Carbohydrates, to stabilize spray-dried proteins, 149
Chemical modifications, 17
Chloramine T, 98
Chromatography, 192
  column, 7
Cleavages, 13
Commercial formulation, 19
  for early preclinical and clinical studies, 19
Commercial formulation development (process), 18
  decisions during, 18–19
  regulatory issues in, 20–22
  timeline, 18
Conformational stability, 51
Cosolvents, preferentially excluded, 66–67
Critical micelle concentration (CMC), 164, 165, 171
Cyclic imide, 13
Cytokine receptor homology (CRH), 30
Deamidation, 13; *see also under* Polypeptides, structure of
Decision tree, role of established for formulation design, 181–182
Degradation
  chemical and physical mechanisms, 160
  conditions used to accelerate, 6
  enzymatic, of non-ionic surfactants, 170
  minimization of routes of chemical, 116–117
Degradation products, qualification of, 17
Dehydration-induced unfolding, mechanism for inhibition of, 123–124
Delivery of products, 16
Denaturants, removal of, 41–43
  solid phase, 43
Denaturation, 13, 70–72
Deuterium, 96–97
Dialysis, 41–42
Dilution, 42–43
Disaccharides, 149
Disaggregation, high pressure, 48
Disulfide bond analysis, 50
Disulfide bond formation, 41

Samsung Bioepis Exhibit No. 1002
Page 7836

Dosage forms
  design of successful, vii–viii
  proper choice of, 183–185
  unconventional, 17
Drug delivery systems, specialized, 185
Drug design, rational, vii
Drying-air volumetric flow rate (v_{da}), 147
Electrophoresis, 7
Equilibrium unfolding, 62
Erythropoietin, recombinant human, 137
Escherichia coli (*E. coli*)
  deamidation of histidine-containing protein in, 90
  recombinant production of native proteins from, 27–28
    cell washing and lysis, 32–34
    distribution of expressed proteins, 28–31
    methods to analyze folded structures, 48–51
    purification and refolding of proteins from inclusion bodies, 36–37
    purification and refolding of soluble, misfolded proteins, 35
    purification of expressed proteins from inclusion bodies, 36–37
    purification of soluble, folded proteins, 34–35
    refolding mechanism, 38–40
      disulfide bond formation, 41
      effects of excipients, 44–47
      effects of tag sequences, 44
      high pressure disaggregation and refolding, 48
      removal of denaturant, 41–43
      response surface methodology, 47–48
Excipients, 21, 170
  animal-derived, 15
  choice of, 117, 120–121, 186–188
  effects on refolding mechanism, 44–47
  interactions with proteins, 65, 66–70
    preferentially excluded cosolvents, 66–67
  limiting solvent accessibility, 96

Fibroblast growth factor (FGF), 31, 32, 69
Fibronectin, 98
Formulation change/amendments, results required to file, 20–22
Formulation development, 1–2, 10; *see also specific topics*
  estimates of resources needed for, 188–189
Formulation development (*cont.*)
  financial ramifications of rapid *vs.* delayed, 110–111
  necessary studies for, 15–16
  preformulation development, 4–5
  preparation for
    resource requirements, 3–4
    useful information for designing formulations, 4
Formulation optimization studies, design of, 15
Formulation options for protein pharmaceuticals, 10
  liquid formulations, 10–11
  single- and multidose forms, 12
  solid dosage forms, 11–12
Formulation problems, strategies to overcome, 17
Formulation studies, information useful for, 4
Formulation variables, optimization of, 13–15
Formulations
  constraints on pharmaceutically acceptable, 182–183
  important components, 13–15
Free energy *vs.* extent of reaction, 73
Freeze-drying microscopy, 115
Freeze-thawing, and stability, 71, 125
Freezing protection, mechanism for, 121–123
Frozen formulations, 183–184
GdnHCl, 35, 36, 45
Gel filtration, 43
Gibbs adsorption equation, 162–164
Glass immobilization hypothesis, 149, 151
Glass transition temperature (T_g), 113–116, 120, 125, 126
Glutathione S transferase (GST), 44
Glycine, 116, 126, 150
Granulocyte colony stimulating factor (G-CSF) receptor, 29
Growth factor
  fibroblast, 31, 32, 69
  keratinocyte, 31, 32, 35
Growth hormone, recombinant human, 137, 143, 147–148, 152–154, 167, 170, 171
Growth hormone-releasing factor (GRF), 90
Handling of products, 16
High-pressure liquid chromatography (HPLC), 192
High throughput formulation (HTF), 178–179, 186, 188, 189
  concept, elements, and structure of, 179–181

Samsung Bioepis Exhibit No. 1002
Page 7837

High throughput formulation (HTF) (*cont.*)
requirements for, 194
software and databases to assist, 179, 189–191
unified strategy for, 194–195
Histidine-containing protein (HPr), 90
Hydrogen to deuterium (H-D) exchange rates, 96–97
Hydrolysis of peptides, 85; see also Deamidation
Hydrophobicity and hydrophobic interactions, 62–63, 72, 95, 161, 163, 166
Hydrophobicity reversal, 167–168
Hydroxyethyl starch, 126
Impurities, 14–15
Inclusion bodies (IBs), 29, 36–37
Infrared (IR) spectroscopy, 191–192
Interleukin-1$ (IL-1$), 28–30
Interleukin 6 (IL-6), 40, 50, 51
I(SH) and I(SS), 39, 41
Isothermal and non-isothermal stress testing, 194
Keratinocyte growth factor (KGF), 31, 32, 35
Lactate dehydrogenase (LDH), 138, 144, 145, 154, 155
Lactose, 149, 150
Ligands, 119–120
non-specific, 96
specific binding, 68–69
Light scattering/turbidity, 7
Liquid formulations, 10–11, 184
excipients for use in, 186–188
Lyophilization-induced protein unfolding, inhibition of, 112–113
Lyophilized products/formulations, 11, 109–110, 184, 188
minimal criteria for successful, 111–117
minimization of routes of chemical degradation, 116–117
storage below formulation glass transition temperature, 113–114
strong, elegant cake structure, 114–116
water content relatively low, 114
rational design of stable, 117–118
bulking agent, 126
choice of buffer, 118–119
ligands/pH optimizing thermodynamic stability of protein, 119–120
trehalose/sucrose to inhibit protein unfolding and provide glassy matrix, 120–126
Lyoprotectants, 149
Lysis buffers, 33
Maltose binding protein (MBP), 44
Mannitol, 116, 126, 137–138, 149–151
Manufacturing process, 21
Metal-catalyzed oxidation (MCO), 94–95
Metals, trace, 94
Methionines and methionine residues, 94–100, 116–117
Micelle concentration, critical, 164, 165, 171
Microcharacterization methods, 7
Monoclonal antibodies: *see* Recombinant humanized anti-IgE monoclonal antibody
Murine IFN-((mIFN-(), 37–38
Mutagenesis, site-directed, 17
OmpA, OmpG, and OmpT proteins, 28–29
Optimization of formulation variables, 13–15
Osmotic pressure, 165
Ovalbumin, 40
Oxidation (processes), 13; *see also* Polypeptides, structure of, role in protein oxidation
excluded effects on, 98–99
metal-catalyzed, 94–95
non-site-specific, 94–97
Oxygen, singlet ($^1O_2$), 93
Packaging material, 21
Parathyroid hormone, 95
Peptides: *see* Polypeptides
Periplasmic secretion strategy, 28–29
Peroxides, organic, 93
pH, 14, 186
Polyethylene glycol (PEG), 46
Polypeptides, structure of, 85–86, 101
deamidation rates and, 86–87, 92
primary structure effects, 87–89
secondary structure effects, 89–91
tertiary structure effects, 91–92
role in protein oxidation, 92–93, 100–101
conformational control of oxidation in aqueous solution, 97–99
effects of oxidation of methionines on protein structure, 95–96
limiting solvent accessibility of residues, 96–97
structural control of oxidation in lyophilized products, 99–100
types of oxidation processes, 93–95
Polysorbate concentration, critical, 153, 154

Samsung Bioepis Exhibit No. 1002
Page 7838

Preferential exclusion mechanism, 122–123
Preformulation development, 4–10
  development of analytical methods, 6, 7
  evaluation of the significance of problems, 7–8
    qualitative assessment, 9–10
    quantitative assessment, 8–9
  information obtained from, 5
Preformulation studies, 185–186
Preservatives, 12
Pressure, and stability, 72
Product development timeline, 18
Protein formulation: *see* Formulation
Protein pharmaceuticals, characterization of, 5
Proteins; *see also specific topics*
  maintenance of biophysical and biochemical properties of, 16
  typical methods used to characterize, 6, 7
Proteolysis, 85
  limited, 51
Purified protein, 3
Purity of raw materials, 14–15
Reaction, free energy *vs.* extent of, 73
Receptor binding, 49
Recombinant human erythropoietin (rhEPO), 137
Recombinant human growth hormone (rhGH), 137, 143, 147–148, 152–154, 167, 170, 171
Recombinant humanized anti-IgE monoclonal antibody (rhuMAbE25), 137–138, 148, 150–151
Regulatory applications, information included in, 20, 21
Regulatory documents, 22–23
Regulatory guidelines, 2
Regulatory license for drug product, results required to apply for, 20
Residues; *see also* Methionines and methionine residues
  asparagine (Asn), 86–92
  solvent accessibility, 96–97
Response surface methodology (RSM), 47–48
Salts: *see* Buffers/salts
SASP (acid-soluble spore proteins), 97–98
Sequence analysis, 190
Serine hydroxymethyltransferase (SHMT), 91
Sodium chloride (NaCl), 118
Sodium dodecyl sulfate (SDS), 160
Solid dosage forms, 11–12
Solubilizer, 14
Solutes, excluded, 99
Solvent accessibility of residues, 96–97
Spectroscopy, 7, 50–51, 191–192
Spray-drying of proteins, 156
  conditions, 140
    drying air volumetric flow rate, 147
    liquid feed rate and atomising air volumetric flow rate, 146–147
    temperature (T_{inlet}, T_{outlet}), 140–146
  developments in last 10 years, 136–138
  equipment, 139–140
  formulated systems, 149–151
  influence of formulation, 147
  pure proteins, 147–149
  reasons for, 135–136
  use of added surface active substances, 151–156
Stability (physical), protein, 61–62, 73, 181
  kinetic control of, 63–65
  physical factors affecting, 70–72
  self-stabilization, 69–70
  thermodynamic control of, 62–63
Stability problems, typical
  causes and solutions, 13
Stability protocols, 193–194
Stability studies, 3
  accelerated, 5–6
  guidelines for, 20
  storage, 15–16
Stabilization, structural, 187
Stabilizers, 14
  non-specific, 96, 187
Storage stability studies, 15–16
Stress testing, non-isothermal, 194
Sucrose, 66, 113, 114, 120–121, 138, 149, 150; *see also under* Unfolding
Sugar; *see also* Sucrose
  rationale for choice of stabilizing, 120–121
Surface denaturation, 13
Surface tension, 165
Surfaces, proteins and surfactants at, 161–166
Surfactant-protein interactions, 159–160
  in solution, 166–167
Surfactants, 188
  effects on protein assembly state, 167–169
  effects on proteins during freezing, freeze-drying, and reconstitution, 169–170
  non-ionic, 160, 161
    enzymatic degradation of, 170

Samsung Bioepis Exhibit No. 1002
Page 7839

Surfactants (*cont.*)
nonionic, to inhibit aggregation, 127
recommendations for protein formulation, 170–171
at surfaces, 161–166

Tag sequences, 44
Temperature, and stability, 70–71
Tetrapeptides, 90
Thermal analysis, 7
Thermodynamic box, classical, 77–78
Timasheff preferential exclusion mechanism, 73–78
Tonicity, 182
Tonicity modifier, 14
Transportation of products, 16
Trehalose, 113, 120–121, 149, 150, 154, 155; *see also under* Unfolding
Trypsinogen, 138, 148
Turbidity, 7
Tween 20, 164–170
a/b-type acid-soluble spore proteins (a/b-SASP), 97–98

Unfolding
dehydration-induced, 123–124
equilibrium, 62
lyophilization-induced, 112–113
trehalose/sucrose to inhibit, 120–126

Water replacement hypothesis, 149, 151
Water replacement mechanism(s), 123–124
Wide-angle X-ray diffraction (WAXS), 150
Wyman linkage function, 73–78

Samsung Bioepis Exhibit No. 1002
Page 7840

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Page 7841

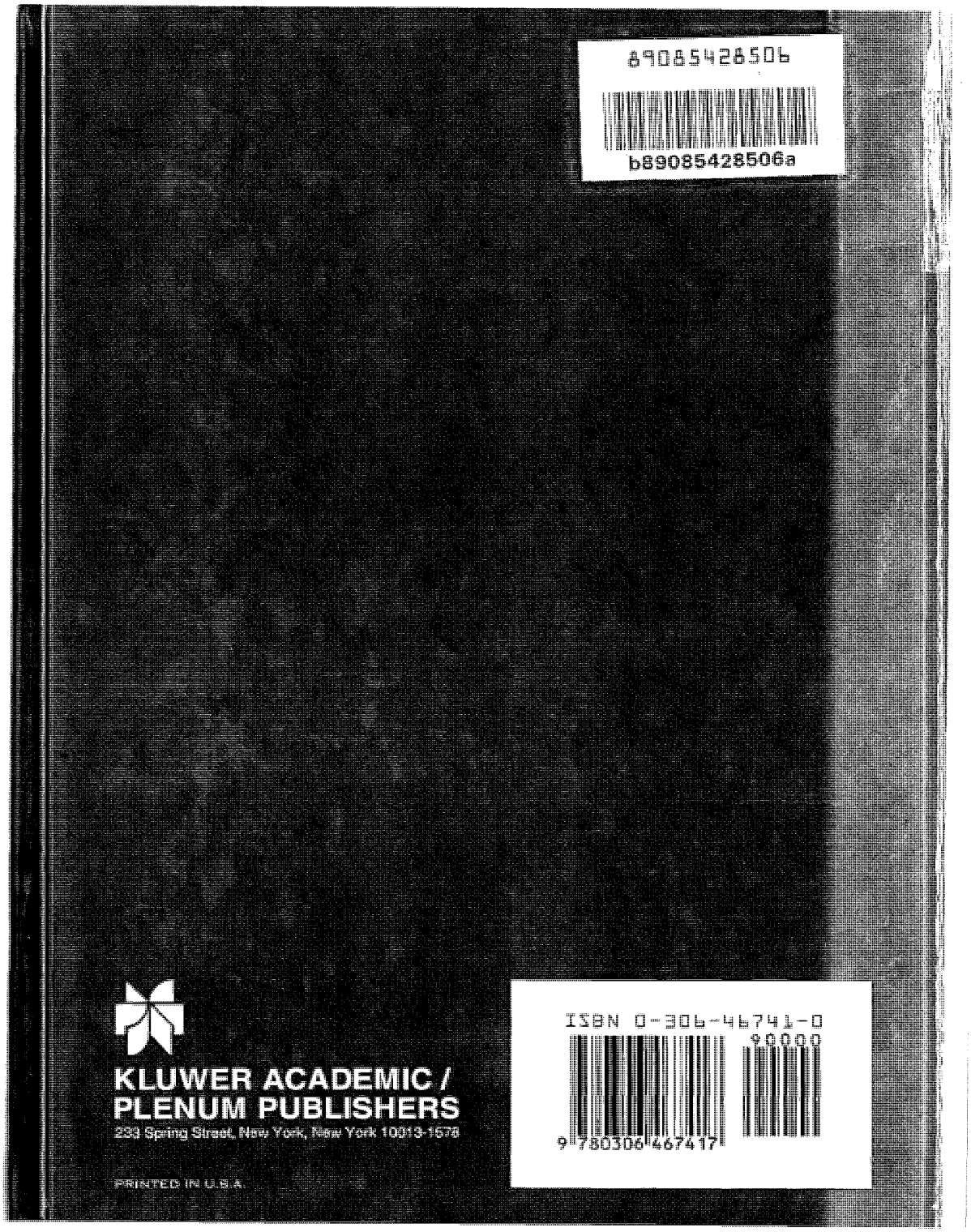
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Page 7842



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Date 29.11.2018

Reference	Application No./Patent No.
198 441 u6/ne OPPO 02	11001632.6 - 1112 / 2359834
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
- ☐ Communication of
- ☒ Oral Proceedings on 21.01.2019

Enclosure(s):
- ☒ Letter from the proprietor of the patent of 21.11.2018
- ☒ Letter from the opponent 01 of 19./21.11.2018
- ☒ Copy (copies) P: claims, AR1,2, cited documents D39-D41
  O1: cited documents D39, D43-D46

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

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☒ **Registered letter**

Samsung Bioepis Exhibit No. 1002
Page 7844

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November 19, 2018

Opposition against European patent EP 2 359 834
(Application No. 11 001 632.6)

Title:	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor:	Alexion Pharmaceuticals, Inc.
Opponent:	Amgen Inc.
Our Ref.:	AMG17203OP

Submission in Preparation of Oral Proceedings Scheduled for January 21, 2019:

This is to confirm that I will attend the Oral Proceedings scheduled for January 21, 2018 on behalf of Opponent 1 (Amgen Inc.).

I expect to be accompanied by Dr. Simon Geiger of my office. I furthermore expect to be accompanied by Tom Wrona, Senior Patent Counsel at Amgen's Patent Department.

Finally, I expect to be accompanied by our technical expert, Dr. Michael Treuheit. Dr. Treuheit's scientific qualifications will be apparent from his declaration (**D23**, Appendix D).

It is respectfully requested that Dr. Treuheit be permitted to make oral submissions during the Oral Proceedings on specific technical aspects of the case, in particular the different aspects of chemical and physical stability of antibody formulations, the content of and the data provided in his Declaration (**D23**), and the content of, and the data provided in the declaration of Dr. Miano (**D33**). Any oral submission by Dr. Treuheit would be made under my responsibility and control, as required by **G 4/95**.

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8 Rechtsanwalt

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PR-Nr. 1278

Samsung Bioepis Exhibit No. 1002
Page 7845

Any submissions made during Oral Proceedings on behalf of Opponent Amgen Inc., including any possible submissions by our experts, will be in English.

Assuming that the Proprietor's and the Co-Opponent's representatives will likewise make their submissions in English, no translation will be required.

Respectfully,

Dr. H. Ulrich Dörries
European Patent Attorney

UD/KS

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Page 7846

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November 21, 2018

Opposition against European patent EP 2 359 834
(Application No. 11 001 632.6)
Title: Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor: Alexion Pharmaceuticals, Inc.
Opponent: Amgen Inc.
Our Ref.: AMG17203OP

In preparation of Oral Proceedings and in Reply to Proprietor's Submission dated March 22, 2018

I. Requests

1. We maintain our request pursuant to Article 101(2) EPC that EP 2 359 834 (hereinafter "the opposed patent") be revoked in its entirety.

2. We further maintain our request for oral proceedings pursuant to Article 116(1) EPC, in case the Opposition Division does not consider the above request justified on the basis of our written submissions.

II. Newly submitted documents

3. We herewith submit the following documents in response to the Proprietor's Observations:

D39 Tacken, P.J. *et al. Blood* **2005**, *106(4)*, 1278-1285;

D40 WO 2004/050017 A2 (Bowdish, K. S. *et al.*, June 17, 2004);

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PR-Nr. 1278

Samsung Bioepis Exhibit No. 1002
Page 7847

D41 WO 2004/108158 A1 (Rother, R. P. *et al.*, December 16, 2004);

D42 U.S. Patent No. 6,407,213;

D43 Emery *et al.*, *Arthritis & Rheumatism* 2006, *54(5)*, 1390-1400;

D44 Rituximab 1997 Product Data Sheet;

D45 Declaration by Prof. Ronald Taylor, including CV (Appendix A); and

D46 Hillmen *et al.*, *N. Eng. J. Med.* 1995, 333:1253-8.

III. Inadmissible Amendments (Art. 100(c) EPC / Art. 76(1) EPC, 123(2) EPC) and Insufficiency of Disclosure (Art. 83 EPC)

4. We have noted the OD's preliminary views expressed under items 6 and 8 of the Preliminary Opinion according to which the requirements of Articles 76(1) and 83 EPC are met. We are grateful for the OD's guidance in this regard, although we respectfully disagree. We shall expand on the issue of compliance with the requirements of Articles 100(b) and (c) EPC in the course of oral proceedings.

IV. No Inventive Step (Article 100(a) EPC / Article 56 EPC)

5. As argued in our Notice of Opposition, **D1** may be regarded as the closest prior art, which was not disputed by the Proprietor. In the alternative, **D2** to **D4**, **D11** and **D18** all represent promising springboards for the claimed subject matter. The allegedly distinguishing features of the claimed subject matter over **D1** are

- the single-use dosage form of 300 mg,
- in a concentration of 10 mg eculizumab/mL
- resulting in a total volume of 30 mL
- wherein the solution is preservative free.

6. The application is silent about supposed technical effects of <u>any of these features</u>. As argued before (sections 36 to 42 of our Notice of Opposition), it is plainly obvious and clearly most convenient for the person of skill in the art to select an amount of 300 mg eculizumab in order to provide a composition that is suitable for the administration of individual doses of 600 mg or 900 mg, as disclosed *inter alia* in **D1** (e.g., [0082], [0088], [0091]). We also enclose a Declaration by Prof. Taylor (**D45**) which confirms this, referring to the example of the use of a single-use dosage form of rituximab of 500 mg for doses of 500 or 1000 mg to treat

rheumatoid arthritis (see **D43** and **D44**). We also note that a similar view was (rightly) taken by the Primary Examiner during prosecution of the opposed patent (Communication dated July 21, 2011, p. 3, 6th paragraph).

7. Further, based on obvious medical considerations, but also regulatory guidelines (**D19**, p. 1/9, 4th and 5th paragraph), the skilled person would avoid using preservatives in multi-dose preparations, whenever possible. Once an amount and concentration of active agent have been determined, the total volume is inherently defined. This leaves the antibody **concentration of 10 mg/mL** as the only possibly relevant distinguishing feature over the prior art.

8. Notably, and in agreement with our position, the Proprietor has focused on discussing the alleged technical effects of the concentration of 10 mg/mL.

9. On a side note, the Proprietor argues that PNH would require that patients start treatment very quickly after having been diagnosed (e.g., sections 4.2.2 and 4.2.3). This allegedly requires that eculizumab must be stored *"on site at a location nearby"* to be ready for use when a patient is identified. It is unclear why the specific *"nature of PNH"* would demand such storage nearby, though. In the absence of any tangible evidence that there is a difference in this regard between PHN and any other disease evoking significant symptoms, this remains a completely unsupported allegation which is contested.

10. Indeed, many of the documents cited in our Notice of Opposition or during prosecution contain general descriptions of PNH and the repercussions the disease has on a patient's life. None of the documents mentions that it would be necessary to start treatment immediately after diagnosis. It seems worth pointing out that PNH is a chronic condition and the treatment with eculizumab aims at improving the quality of life of afflicted patients (see, e.g., [0030] of **B1**). The therapy does not aim at curing an acutely life-threatening condition. Accordingly, trials described in the prior art commonly involved observation periods of up to 13 weeks after identification of eligible patients and before start of the treatment (cf. **D18**, p. 1234, right column 1st and 3rd full paragraph; but also [0070] of **B1**). This would hardly appear to be ethically justified if treatment would indeed have to start immediately, as purported by the Proprietor. We also note that **D18** reports the need to vaccinate PHN patients prior to treatment against *N. meningitides* (page 1234, right column, end of 2nd paragraph), again indicating that treatment was not initiated immediately upon diagnosis. Further, we refer to new reference **D46** (Hillmen, enclosed herewith), whose authors followed a group of 80 PHN patients (see Abstract) for up to 48 years and reported a median survival of 10 years (see the Discussion). None of the statement in this article indicates that PHN patients, once diagnosed, would need more immediate treatment than other chronically ill patients. The Proprietor's

allegations in this regard are thus not only unsupported but also contrary to the facts emanating from the documents on file.

11. Any requirements derived from this alleged need for immediate treatment (such as stability over 48 months) must therefore be rated as being artificially construed. The requirements a pharmaceutical composition suitable for use in the treatment of a patient suffering from PNH would have to meet are no different from the requirements pharmaceutical compositions for the treatment of other chronic diseases would have to satisfy.

IV.1 The concentration constitutes an arbitrary choice

12. We maintain that the concentration of 10 mg/mL, presented by the applicant as allegedly providing "the ideal balance" of properties (e.g. section 5.3.2, 5.3.10, 5.4.8, etc. of the submission), constitutes an arbitrary choice among numerous equally (or possibly even more) suitable alternatives.

13. It is established case law that a feature which is considered an arbitrary choice among equivalent variations cannot contribute to inventiveness (**T 206/91** (item 5.5) and **T 37/82** (item 3).

14. In this regard, we first of all note that the concept of "the *ideal balance of properties"* advanced by the Proprietor should be seen for what it is: a red herring which has been artificially created in the hope to successfully argue for an allegedly unobvious technical effect resulting from meeting said artificial (and wholly arbitrary) requirement. The suggestion under item 5.3.2 of the Observations that the allegedly "ideal" balance purportedly achieved by the claim features would have been instrumental to *"the approval of the claimed composition as a treatment for PNH"* has no basis whatsoever. There is no evidence that the regulatory bodies specifically preferred the "balance of properties" offered by the approved formulation over the "balance of properties" displayed by any other formulation. In fact, there is no such thing as an objectively "ideal balance" of properties of any pharmaceutical, since any "balancing" will be based on subjective elements and preferences.

15. As an aside, the approval obviously did not relate to *"the claimed composition"* but to an antibody that has been recombinantly produced in specific cells following a specific manufacturing and purification protocol, and which has been formulated in specific buffers and excipients, and at a specific pH. None of this is mentioned in the claim. There is no evidence that the "balance of properties" displayed by the approved product is shared by each and every formulation falling within the claim; rather, the contrary is to be assumed, as will be explained further below.

16. According to a more realistic proposal by the Proprietor, the underlying problem of the original filing lies in the provision of a(n alternative) useful treatment for patients suffering from PNH (section 5.10.2 of the submission). If anything, a pharmaceutical composition to serve this purpose must be suitable (or useful) for use in the treatment of patients suffering from PNH. Any requirement going beyond this (such as stability during storage over 48 months, maintaining over 98% monomer content, etc.) is – in the absence of any evidence to the contrary – arbitrary, and thus, cannot be invoked as justification for an inventive step.

17. With regard to the 98% monomer threshold we further note that according to **D20**, the standard of the World Health Organization (WHO) for commercial intravenous immunoglobulin products was an aggregate level of less than 5% (**D20**, p. 9, left column, 1st paragraph). This means that, according to the WHO, any antibody solution comprising at least 95% antibody monomer would be suitable for intravenous delivery and could thus be released into the market. It seems obvious that the Proprietor tries to generate an artificially high barrier to make an insignificant difference (if there is any) appear significant. The 98% monomer threshold is therefore not technically meaningful.

18. The Proprietor also failed to provide any evidence that would show that a monomer content of 98% was essential for maintaining biologic activity in an antibody formulation. As noted above there is no evidence that this exact threshold is technically relevant. Why would 96% or 95% monomer content be insufficient? Perhaps more importantly, there is no evidence of a correlation between aggregation and loss-of-activity for this specific antibody! This is even more relevant for the alleged requirement of stability for 48 months. It is apparent from the Proprietor's own allegations that Soliris® obtained market approval by the FDA with a shelf life of 24 months, as will be discussed in more detail below. This means that both, the Proprietor and the regulatory authorities considered a formulation with a shelf life of 24 months, when properly stored, to be sufficiently stable for use in the treatment of PNH.

19. In fact, the concentration of 10 mg/mL cannot serve to distinguish the claimed subject matter from the prior art for the purposes of Article 56 EPC, as there is no particular technical effect conferred by this specific concentration. This is *inter alia* evident from **D23** and **D25** and even from the Proprietor's own data.

20. **D23** presents data on solutions of eculizumab in the buffer which is used for the commercial formulations of the drug, using three different analytical methods, size-exclusion ultra high-performance liquid chromatography (SE-UHPLC), anion exchange high-performance liquid chromatography (AEX-HPLC), and reduced sodium dodecyl sulfate capillary electrophoresis (rCE-SDS). These methods allow for assessing both chemical and structural stability of an antibody solution.

21. The Proprietor has argued that the data presented with the declaration by Dr. Michael Treuheit may not be informative, as stability of the solution was tested *"only over a 13 week period"*, which would be significantly less than the 48-month stability allegedly demonstrated in **D33**. We note in this regard that the Proprietor (and his party expert Dr. Miano) has at the same time admitted that stability testing at elevated temperatures would provide a good indication of effects which might be seen at 2 to 8°C over a longer period of time (see section 5.3.5 of the submission). This is consistent with the statement of Dr. Treuheit (cf. section 4 of **D23**). The data presented in **D23** for elevated temperatures thus provides a good estimate of the behavior of the tested antibody solutions over a longer period of time than the tested 13 weeks.

22. The Proprietor finds the data presented in **D23** to be in line with the data submitted as part of the Miano declaration (**D33**), by selectively interpreting the data to suit his line of arguments. This selective and self-serving interpretation is, however, misleading.

23. Antibody degradation occurs via multiple pathways, some of which are interdependent, such as denaturation, aggregation, surface adsorption, chemical degradation (oxidation, deamidation, formation of acidic species), etc. This has been correctly noted by the Proprietor under section 4.3.3 of the submission. One particular degradation pathway does not necessarily dominate for any one antibody (see also section 4.3.4 of the submission), however, Dr. Miano has solely analyzed aggregation of the antibody at merely two different concentrations (cf. section 3 of **D33**). **D23**, in contrast, provides data on aggregation (SE-HPLC, rCE-SDS) and chemical degradation (AEX-HPLC, rCE-SDS) for five different concentrations. The rCE-SDS analysis, in particular, provides a good overview over different degradation pathways, as it allows for separation of both chemically and structurally altered species with very high resolution (cf. section 7 of **D23**).

24. A closer look at the data of **D23** reveals that all tested concentrations of eculizumab (i.e., 2, 5, 10, 30 and 50 mg/mL) were equally stable at 4°C over the tested period, as measured by SE-HPLC (**D23**, Appendix A). At elevated temperature (25°C), there is slight aggregation visible for 30 mg/mL and 50 mg/mL, but this is at levels that are hardly significant. The lower concentrations (10, 5 and 2 mg/mL) show levels of monomer at the latest time point that are higher than at the earliest time point. This may be taken as an indicator of experimental noise in the measurement and the observed decrease in the monomer content at 30 mg/mL and 50 mg/mL is well within these limits.

25. In any case, all concentrations show a monomer content that is above 98% at any time point and would thus be suitable for release into the market according to the (we believe arbitrary) limits defined by the Proprietor (section 3 of **D33** and e.g. sections 5.3.4, 5.3.6 of the submission) and even more so within the limit defined by the WHO (95% monomer). The

Proprietor alleged that the 30 mg/mL and 50 mg/mL concentrations would fall *"below the release parameter for commercial lots of eculizumab"* (section 5.4.5 of the submission), but this is clearly incorrect, as all tested solutions exhibit a monomer content that is comfortably above the 98% threshold at any time point.

26. In addition, the lower concentrations of 5 and 2 mg/mL exhibit a monomer content at the latest time point that is higher than for the 10 mg/mL solution. Thus, if one follows the interpretation of the Proprietor and takes these very small differences into account, 10 mg/mL is actually less stable than either 5 mg/mL or 2 mg/mL and thus obviously not the "ideal" concentration. If the observed increase of monomer content for the 5 mg/mL and 2 mg/mL is interpreted as noise, they are at least as stable as the 10 mg/mL solution, i.e., the concentration of 10 mg/mL is of no advantage.

27. We note at this point that the commercial formulation of eculizumab is diluted to a concentration of 5 mg/mL prior to administration to the patient (section 5.3.9 of the submission and **D24**, p. 2 under *"Preparation and Administration"*), which means that the higher concentration of 10 mg/mL is, in any case, of no benefit to the patient, when compared to 5 mg/mL. The infusion volume (and thus infusion time) would be identical no matter if the antibody was stored at a concentration of 10 mg/mL or 5 mg/mL (or alternatively 6, 7, 8 or 9 mg/mL).

28. When looking at the chemical degradation, as measured by AEX-HPLC in **D23** (Appendix B), there is, again, no significant difference between the tested concentrations. Slight degradation is visible for all samples at elevated temperatures, but no discernible advantage of any concentration is apparent. If anything, when taking minimal changes into account as the Proprietor did, the solution of 30 mg/mL shows slightly less chemical degradation at the latest time point (25°C), compared to 10 mg/mL. In addition, the AEX-HPLC analysis also makes clear that chemical degradation appears to be the more relevant degradation pathway for eculizumab, as all samples show markedly faster degradation than in the SE-HPLC assay.

29. The overall picture (chemical and physical degradation), as determined by rCE-SDS is in accordance with this analysis. If anything, the higher concentrations of 50 mg/mL and 30 mg/mL are slightly more stable than 10 mg/mL (purity at the latest time point) at 4°C. At 25°C some degradation takes place in all samples, but there is no difference between 10, 30 and 50 mg/mL. If anything, 5 mg/mL is slightly worse than the other concentrations.

30. In summary, **D23** shows that, leaving experimental noise aside, there is no difference in stability between solutions of eculizumab, formulated in the commercial buffer at concentrations of 2, 5, 10, 30 and 50 mg/mL. Even if the OD was inclined to take the minimal differences into consideration that are visible in this raw data, there is no clear picture. All

concentrations are well within the specifications purported by the Proprietor (at least 98% monomer content) and 10 mg/mL shows, if anything, slightly faster chemical degradation than 30 mg/mL. The concentration of 10 mg/mL provides no advantage, and hence provides no technical effect that may be taken into account for an assessment of inventive step.

31. According to the Proprietor, the Miano declaration (**D33**) shows the benefits of the concentration of 10 mg/mL. We note that **D33** greatly exaggerates entirely insignificant differences to make a minimal effect appear technically relevant (see in particular the scale on the y-axis). In addition, **D33** only analyzes two concentrations, 10 mg/mL and 30g/mL and therefore cannot serve as evidence that 10 mg/mL is in fact "ideal", or even just better than concentrations of, e.g., 25, 15, 9, 8, 5 mg/mL, etc. Two data points are obviously insufficient to draw such a conclusion.

32. As explained by the Proprietor, the initial shelf life of commercial formulations of eculizumab was determined to be 24 months, which was later extended to 30 months, when stored at 2°C to 8°C (see FDA letters annexed to **D33**). Any discussion relating to stability of the "bulk" drug prior to final formulation is immaterial, as no feature relating to repackaging of bulk product is recited in the claim as granted. This means that both the Proprietor and the regulatory agencies found a shelf life of 24 months to be sufficient for Soliris® to be approved and enter the market at the time the opposed patent was filed.

33. More importantly, as the current shelf life is specified to be 30 months, any final eculizumab drug product older than 30 months would have to be discarded. No responsible physician would administer eculizumab compositions older than the specified shelf life (**D24** at p. 6, under *"How Supplied/Storage and Handling"* explicitly states the obvious: *"Do not use beyond the expiration date stamped on the carton."*). It is thus entirely immaterial, if the composition claimed by the opposed patent is hypothetically stable for 36, 48, or even 100 months. Any stability beyond 24 months, the shelf life which Soliris® was initially approved with, or at most 30 months, makes no difference whatsoever in the context of the original disclosure.

34. Hence, any solution of eculizumab providing sufficient stability over 24 months, when stored properly, would be equally suitable for use in the treatment of patients suffering from PNH. The data provided in **D33** shows that both tested solutions provide this degree of stability (see Fig. 2 of **D33**). Even when the required shelf life is extended to 30 months, Fig. 2 of **D33** shows that both, 10 mg/mL and 30 mg/mL, exhibit a monomer content that is distinctly above 98% after 30 months of storage (and, of course above 95%). This means there is no technically relevant difference between the two concentrations, even if the arbitrary 98% threshold purported by the Proprietor was accepted as a measure.

35. If one assumes a correlation between antibody concentration and aggregation tendency (which the person of skill would do, as further discussed below), this would also be true for concentrations of, e.g., 25, 20 and 15 mg/mL and even more so for concentrations between 10 and 5 mg/mL. As noted above, Soliris® is diluted to 5 mg/mL prior to administration, so reducing the concentration to 5 mg/mL would not be detrimental to the patient in any way.

36. This means that both tested concentrations (and the hypothetical alternatives) are equally suitable for the provision of a solution of eculizumab for use in the treatment of PNH. Even if there were slight differences with regard to the formation of aggregates, they would be insignificant in the context of the original disclosure (and any technical problem retrospectively derived from it), which relates to methods of treatment for PNH.

37. Again we note as an aside that Dr. Miano solely analyzed aggregation and only by one particular analytical method. As explained above, aggregation is only one of numerous different degradation pathways that may affect the activity of an antibody drug. Concluding from this very limited data that 10 mg/mL would provide an "ideal" concentration, in particular with regard to biologic activity (sections 4.2.3, 4.3.1, 4.6.9 of the submission) is complete speculation. It is perfectly possible and, when taking **D23** into consideration, indeed not unlikely that chemical degradation at 10 mg/mL occurs at least as fast as at 30 mg/mL. In any case, biological activity was not tested by Dr. Miano and it is thus readily conceivable that the solutions lost their biologic activity without aggregating.

38. The Proprietor thus failed to submit any evidence that the claimed pharmaceutical composition actually provides any particular effect that is relevant in the context of providing a pharmaceutical composition for use in the treatment of PNH.

39. Lastly, **D25** represents further evidence that there is no actual difference in the stability of eculizumab when stored at concentrations between 10 mg/mL up to more than 100 mg/mL. **D25** is a patent application, filed by the Proprietor of the opposed patent and directed to high concentration formulations of anti-C5 antibodies, particularly eculizumab. The Examples of **D25** disclose a wealth of data regarding numerous criteria of antibody stability for eculizumab (**D25**, [0229]) solutions at concentrations of *inter alia* 10, 30 and 105 mg/mL (**D25**, [0244] to [0265]).

40. In section 5.4.6 of the submission, the Proprietor alleged that there was no indication of the relative stability of high concentration formulations compared to the claimed 10 mg/mL composition in **D25**. This is plainly incorrect. Solution V of **D25** is a solution of eculizumab in a buffer comprising 10 mM sodium phosphate, 150 mM sodium chloride and 0.02% polysorbate 80, at pH 7.0 (**D25**, [0262] to [0265]). This composition is identical to the commercial formulation of Soliris® (cf. section 2 of **D33**).

41. This solution is tested against four other solutions comprising eculizumab in concentrations of either 30 mg/mL or 105 mg/mL with regard to, e.g., appearance and osmolality, SDS-gel band pattern, biologic activity but also monomer content via SE-HPLC (**D25**, table 4). The analysis shows that all tested solutions exhibited a monomer content of at least 98% after storage for 24 months at 2 to 8°C, including 30 mg/mL in two different buffers (see also [0282] of **D25**). Thus, all tested solutions would comply with the requirements formulated by the Proprietor after storage for 2 years. There is no significant difference between any of the tested solutions with regard to monomer content. This is equally true with regard to biologic activity as is evident from Table 6 of **D25** (see also [0306]). The authors conclude that

> *"These results indicate that the formulations described herein maintain the functional as well as structural stability of the anti-C5 antibodies dissolved therein for at least 2 years at 2°C to 8°C."* (**D25**, [0306])

42. In summary, **D25** shows that eculizumab may be formulated at a range of different concentrations, particularly at 30 mg/mL and higher, with very good stability over at least 24 months. Moreover, **D25** shows that there are no meaningful differences in terms of stability and biologic activity between solutions at 10 mg/mL, 30 mg/mL and 105 mg/mL. With regard to stability of the antibody, any one of the solutions disclosed in **D25** would thus be equally suitable for being used in the treatment of patients suffering from PNH.

43. The Proprietor stated that the buffer was changed in **D25**, when moving from the stock solution of 10 mg/mL to the higher concentrations and the different solutions would therefore not be comparable (section 5.4.6 of the submission). Notably, the granted claim contains no limitation with regard to the buffer. This means that the purported technical effect ("ideal" stability) must be provided by the allegedly distinguishing feature (i.e., the concentration of 10 mg/mL) over the whole scope claimed (i.e., in any buffer, at any pH, etc.).

44. Further, if differences in the buffer would have a significant impact with regard to long term stability, which the Proprietor appears to suggest, it would be *prima facie* not credible that the technical problem (or technical effect) put forward by the Proprietor is solved (or achieved) over the whole scope claimed (again, this is also discussed in more detail below).

45. In the present case, the Proprietor explicitly relies on the concentration of 10 mg/mL as the sole feature allegedly providing the solution to the technical problem. The technical effect must therefore be provided by the concentration only and not depend on further features that are not recited in the claims (such as choice of buffer, excipients, pH, etc.). The solutions disclosed in **D25** are therefore perfectly comparable to the formulation as claimed, no matter which buffer was used. **D25** shows that the concentration of 10 mg/mL cannot serve as a

distinguishing feature, as it does not provide any technical effect over the other tested concentrations.

46. Notably, **D25**, which is specifically concerned with high concentration formulations of *inter alia* eculizumab and was filed by the Proprietor of the opposed patent, makes no note whatsoever of any challenges or unusual properties of the antibody arising from its allegedly unique and unusual structure. This sheds considerable doubt on the Proprietor's allegations in this regard.

47. In summary, the concentration of 10 mg/mL provides no relevant technical effect over other concentrations (such as 30 mg/mL), and therefore constitutes an arbitrary choice.

IV.2 The skilled person would arrive at the claimed solution without inventive activity

48. Even assuming a concentration of 10 mg/mL would provide any superior stability of eculizumab over other concentrations, which we contest, the skilled person would arrive at this concentration without inventive activity.

49. Starting from **D1**, the person of skill in the art would essentially be confronted with the problem of identifying a suitable concentration for eculizumab in a pharmaceutical composition for use in the treatment of PNH. Based on common general knowledge (such as **D20**, **D21**), the skilled artisan would be aware that antibodies are commonly formulated for intravenous administration at concentrations ranging from 1 mg/mL to 50 mg/mL (**D21**, table 1) or 0.5 mg/mL to 100 mg/mL (**D20**, table 1).

50. From **D22**, the skilled person would furthermore be aware that specifically eculizumab may be formulated at concentrations between 1 mg/mL and 30 mg/mL (**D22**, Fig. 10 and par. [0170] to [0172]). The Proprietor argued that the skilled artisan would not consider **D22**, as it relates to formulations for nebulization. While we agree that formulations for different routes of administration may be different in certain aspects, factors such as long term stability of the active substance and maintenance of biologic activity are clearly relevant for formulations for any route of administration. Thus, in the absence of any alternative guidance as to which concentration would be suitable for the specific antibody in question, the skilled person would take **D22** as an indication that eculizumab, formulated at a concentration of 1 to 30 mg/mL, would at the very least be sufficiently stable and biologically active to be used as a drug.

51. Importantly, even the buffers disclosed as being suitable in **D22** encompass the buffer used in the commercial formulation of eculizumab (Fig. 10, **D22**: 10 mM sodium phosphate, 150 mM sodium chloride, pH 6-8 and 0.001 to 0.02% Tween 80, compare section 2 of **D33**). "Tween 80" is synonymous for polysorbate 80 (see also [0069] of **D22**).

52. **D22** also discloses that eculizumab maintains its integrity after being formulated according to the teaching of **D22** and subsequent nebulization, as shown by SDS-PAGE and HPLC ([0173] of **D22**).

53. Thus, when faced with the task of identifying a concentration for eculizumab in a pharmaceutical composition for the treatment of PNH, the skilled person would not disregard **D22**, as it shows that eculizumab may be formulated at a concentration of 1 to 30 mg/mL while maintaining its structural integrity, i.e., without aggregating or precipitating.

54. The Proprietor tries to create the impression that **D22** would solely teach eculizumab formulations of 30 mg/mL and therefore teach away from the claimed subject matter (section 5.8.2 of the submission). This is evidently not the case. Fig. 10 of **D22** clearly shows that the concentration of the antibody may range from 1 mg/mL to 30 mg/mL, which obviously encompasses the claimed concentration of 10 mg/mL. Further, paragraphs [0171] and [0172] of **D22** teach that the *"Mab"* referred to in Fig. 10 is eculizumab.

55. Since eculizumab is a humanized monoclonal antibody with a hybrid IgG2/IgG4 constant domain, the skilled person would also readily refer to **D42** (entitled "Method for Making Humanized Antibodies"; submitted herewith) for guidance regarding concentrations. As noted by Prof. Taylor in **D45**, **D42** states that such humanized *"antibodies will typically be formulated … at concentrations of about 1 mg/ml to 10 mg/ml"* (**D42**, col. 45, li. 57, to col. 46, li. 2). **D42** relates to methods for making and using humanized antibodies, including antibodies with hybrid constant domains (**D42**, col. 8, ll. 47-51). Thus, selecting 10 mg/ml as concentration for a humanized mAb with a hybrid IgG2/IgG4 constant domain, such as eculizumab, would have been a typical, arbitrary choice among known concentrations.

56. We therefore submit that starting from **D1**, in combination with either **D22** or **D42** the skilled person would have had a reasonable expectation of success that a pharmaceutical composition comprising eculizumab in a concentration of 10 mg/mL would be useful in the treatment of PNH.

57. At the very least, the skilled person would have adopted a "try-and-see" attitude. We again refer to Board of Appeal decision **T 1241/03**. This case is indeed highly similar to the present one. The patent in suit *inter alia* related to *"aqueous liquid hGH formulations which are stable during long-term storage, namely 6 to 18 months at 2-8°C"* (**T 1241/03**, point 24 of the reasons, emphasis added). A document cited in the proceedings (document (7)) disclosed stable aqueous protein solutions, comprising *"growth and differentiation factors"* (point 28 of the reasons), i.e., not even the precise protein the patent in suit related to. Still, the competent Board found that

> *"[…] the skilled person, although knowing that the development of liquid stable protein formulations is technically unpredictable and does not allow to rely on stability predictions obtained from other, different samples, would not have been deterred from testing the stability of an hGH containing solution comprising the components indicated in document (7).*
>
> *Obviousness is not only at hand when the results are clearly predictable but also when there is a reasonable expectation of success (cf. decision T 149/93 of 23 March 1995; point (5.2) of the reasons). A reasonable expectation of success does not require certainty (cf. decision T 338/97, of 7 February 2000; point (14) of the reasons).*
>
> *Thus, in spite of the understandable uncertainties which always characterize experiments using biologic compounds like proteins, the skilled person had no reason to adopt a sceptical attitude. He/she would have had either some expectations of success or, at worst, no particular expectations of any sort, but only a "try and see" attitude, which - as pointed out in decisions T 333/97 of 5 October 2000; point (13) of the reasons - does not equate with the absence of an reasonable expectation of success (cf. decision T 1045/98 of 22 October 2001; point (17) of the reasons)."*
> (**T 1241/03**, point 31 of the reasons, emphasis added)

58. This approach has been endorsed in further Board of Appeal decisions, such as **T 1149/09** and **T 499/09**.

59. The Proprietor argued that formulation of antibody drugs was regarded *"at the time"* as being a highly complex and unpredictable issue. We note in this regard that **T 1241/03** was concerned with a patent that had a priority date in 1992, i.e., 15 years before the filing date of the opposed patent. Obviously, a task that was regarded to be within the scope of routine experimentation for a skilled artisan in 1992 would have been routine experimentation 15 years later.

60. The Proprietor has further argued that *"a "try and see" situation is considered by the Boards to be limited to particular situations, i.e. where the prior art clearly envisages a specific feature, and the skilled person merely has to determine by routine tests whether such feature does indeed provide a desired effect."* (section 5.5.4 of the submission, emphasis added). This is in fact exactly the case here. **D22** describes formulations of the same antibody (eculizumab) at concentrations ranging between 1 and 30 mg/mL, even in the same buffer. All the skilled person had to do was test a number of concentrations falling within the range disclosed in **D22** (in the very same buffers disclosed in **D22**) and determine their suitability, thereby immediately arriving at concentrations such as 10 mg/mL. Similar considerations apply to **D42**.

61. The Proprietor has stressed that it is essential to consider what the skilled person would have done instead of considering what the skilled person could have done (sections 5.5.1 and 5.5.2 of the submission). Indeed, the skilled person would have been aware of the inherently empirical nature of the task at hand, namely identifying (a) suitable concentration(s) for eculizumab. With this in mind, the skilled person would have looked for any prior art that would indicate a suitable starting point or springboard, which would have undoubtedly led to **D22**. The Proprietor argued that the skilled person would disregard **D22**, but if the alternative would be merely guessing a starting point for the empirical analysis it seems much more likely that the skilled person would have considered the teaching of **D22**, as it teaches stable pharmaceutical formulations of the same antibody. The Proprietor failed to provide any convincing argument why the skilled person would have disregarded **D22**. Further, the skilled person would have alternatively readily considered **D42** for the reasons explained above.

62. Thus, in view of the prior art, the skilled person would have envisaged a range of concentrations (1 to 30 mg/mL) and would subsequently have determined by routine testing which of said concentrations had the desired effect. When testing a limited number of concentrations in the relevant range, for example 1, 10, 20 and 30 mg/mL, and without modifying the teaching of **D22** in any way (i.e., in the same buffer) the skilled person would have instantly arrived at the claimed concentration of 10 mg/mL. No further modification would have been necessary. Alternatively, **D42** would have led the way to the claimed concentration.

63. The skilled person would have been further guided towards the alleged invention by his/her common general knowledge, particularly the known fact that aggregation propensity generally increases with concentration. The authors of review article **D20**, for example note that

> *"Increasing the concentration of antibodies often increases the aggregation tendency of the protein"* (**D20**, p. 9, left column, 2nd full paragraph).

While this differs from the Proprietor's interpretation of the disclosure of **D20** (cf. section 4.6.8 of the submission), the above quote from the document shows that the authors of **D20** would not share the Proprietor's view.

64. Thus, when testing concentrations within the limited range of 1 to 30 mg/mL disclosed in **D22**, the skilled person would have had a clear motivation and incentive based on the common general knowledge to not only test eculizumab at 30 mg/mL, but also at lower concentrations in the expectation of being able to reduce any aggregation tendency that might be found at 30 mg/mL.

65. Thus, the allegedly inventive feature of a concentration of 10 mg/mL would have been arrived at in an obvious manner by following routine experimentation.

66. The Proprietor tried to explain at some length why finding a suitable concentration for eculizumab was a tedious task, necessitating an inventive activity. First, the Proprietor suggested that the formulation of antibodies was a *"complex challenge"* (section 4.3 of the submission). In the absence of any features in the claim further defining the formulation we note that the only challenge the skilled person was faced with in the current case was the identification of suitable concentrations within the range of 1 to 30 mg/mL disclosed in **D22**, (or the range of 1 to 10 mg/ml disclosed in **D42**, for that matter) which would have been achieved (and the claimed solution thereby arrived at) by means of routine experimentation.

67. This is not changed by the fact that *"each antibody is unique"* (section 4.4 of the submission). Even if this was the case, it does not render finding a suitable antibody concentration inventive. Finding a suitable concentration belongs to the steps which are routinely undertaken when identifying a useful formulation for a specific antibody. According to established case law, work *"involving mere routine experiments, such as merely conventional trial-and-error experimentation without employing skills beyond common general knowledge, lack[s] inventive step (**T 455/91**, **T 104/92**)"* (Case Law of the Boards of Appeal, 8th Edition, section 9.18.5). It is not apparent which skills beyond the average and which insights going beyond mere common general knowledge would have been involved in finding suitable (or even the claimed allegedly *"ideal"*) concentration(s) for eculizumab.

68. Notably, many passages of the Proprietor's observations relate to the identification of, e.g., the right *"formulation components"* (section 4.4.1 of the submission), pH value (section 4.4.2 of the submission), *"pH and excipients"* (section 4.5.1 of the submission), lyoprotective additives (ibid.), and so on. Importantly, however, no features related to such parameters are recited in the granted claim. The Proprietor tries to draw a picture of the skilled person being confronted with a complex, multi-dimensional problem. This attempt must fail already in view of the lack of corresponding features of the claim of the opposed patent, and the lack of evidence of an ambitious technical problem having been solved. On the contrary, the skilled person would have been confronted with a rather straightforward task, namely the identification of suitable concentrations. Of course, the determination of suitable concentrations would have to be performed in any case, as the skilled person anyway would have to make a choice in this regard (and would be able to rely on simple and routine measures in this regard) when formulating an antibody.

69. With regard to antibody concentrations used in earlier formulations (section 4.6 of the submission, also section 47 of our Notice of Opposition), the Proprietor argued that the concentration of 10 mg/mL would not clearly emerge as the single most common concentration for pharmaceutical antibody formulations prior to the filing date of the opposed patent. This is, however, not necessary for a finding of obviousness. As noted above,

obviousness is not only at hand when the results are clearly predictable, but also when there is a reasonable expectation of success (**T 149/93**), which does not require certainty (**T 338/97**). This of course means that it is equally not necessary that a skilled person is able to predict a suitable formulation from his/her common general knowledge (section 4.6.7 of the submission). It is similarly immaterial that *"[t]here is no single technique or set of excipients and buffer/pH conditions, which formulation experts have at their disposal, which is guaranteed to lead to stability of any particular antibody product"* (section 4.6.8 of the submission, emphasis added).

70. As correctly summarized by the Proprietor, an observation that can be made based on the antibody products on the market before the filing date of the patent is that *"[t]hose products that can be stored in solution form include five products having a concentration from 1 to 2 mg/ml; four products having a concentration of 10 mg/ml; and three products having a concentration of 20 mg/ml or above"* (section 4.6.3 of the submission). Thus, even assuming 10 mg/mL was not the single most used concentration, it was undoubtedly a very common one. It is not apparent what would have deprived the skilled person of his/her reasonable expectation of success regarding the stability of eculizumab when being formulated at a concentration of 10 mg/mL, particularly not when taking the teaching of **D22** into consideration, which is specific for eculizumab.

71. Finally, the Proprietor puts heavy emphasis on the allegedly unique structure of eculizumab, which supposedly made finding a suitable concentration more challenging, compared to other antibodies. As noted above **D22** specifically discloses suitable concentrations for eculizumab and therefore any (anyway completely hypothetical and speculative) alleged problems due to the uniqueness of the antibody are irrelevant when taking **D22** as prior art into consideration. It further seems worth pointing out that the application as filed does not mention any challenges arising from the structure of eculizumab either. To the contrary, the original disclosure merely states that

> *"Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed. (1985)."* (**WO**, p. 22, li. 21/22)

The application as filed thus confirms that eculizumab may be formulated according to common procedures. It was also known prior to the filing date of the opposed patent that eculizumab comprises a chimeric IgG2/IgG4 constant region, as evidenced by **D39**, submitted herewith (**D39**, p. 1279, left column, under *"Recombinant antibodies"*). Contrary to the Proprietors allegation, there are other antibodies disclosed in the prior art which contain the same engineered heavy chain constant region (see **D15**, e.g., p. 8, point 9); **D16**, throughout

the document, e.g., p. 5, 10, 11, etc.; but also **D40**, SEQ ID 67 and **D41** p. 18, submitted herewith).

72. None of these documents mentions any complication arising from the structure of the heavy chain constant region, or teaches that the IgG2/IgG4-containing antibody would behave differently when formulating them from other antibodies. To the contrary, all of them contain statements that the disclosed antibodies may be formulated according to standard procedures (**D15**, p. 24, 3rd full paragraph; **D16**, p. 17/18; **D40**, paragraph bridging p. 36/37; **D41**, p. 16). As noted above, this is equally true for **D25**, which specifically relates to high concentration, long-term stable formulations of anti-C5 antibodies, such as eculizumab. Eculizumab was used in all examples of **D25**. Yet, **D25** makes no mention of any difference between eculizumab and other anti-C5 antibodies which would be due to its allegedly unusual heavy chain.

73. Notably, all of the above-mentioned patent applications **D15**, **D16**, **D25**, **D40** and **D41** were filed by Alexion, the Proprietor of the opposed patent.

74. Thus, based on the state of the art at the filing date of the opposed patent, there was nothing to suggest that eculizumab would require conditions for its formulation that would be significantly different from commonly used conditions. It is therefore not apparent that the skilled person would have been discouraged from testing commonly used concentrations and confirming the expected usefulness of such concentrations, including the concentration as claimed. Indeed, the concentration which according to the Proprietor is "ideal", can by no means be considered to be unusual.

75. In summary, the claimed subject matter is wholly obvious in view of **D1** in combination with **D22** (or **D42**) and/or common general knowledge.

IV.3 <u>The technical problem as formulated by the proprietor is not derivable from the application as filed and has in any event not been credibly solved</u>

76. When justifying the reformulation of the technical problem, the Proprietor pointed to the Guidelines of Examination (section 5.10.1 of the submission), which state that it may be possible to rely on new effects *"submitted subsequently during the proceedings by the applicant, provided that the skilled person would recognize these effects <u>as implied by or related to the technical problem initially suggested</u> (see G−VII, 11 and T 184/82)"* (emphasis added). Under section 5.10.3 of the submission, the Proprietor then recites the example at G-VII-11 of the Guidelines where pharmaceutical activity and toxicity are considered to be sufficiently related for the technical problem to be reformulated accordingly. However, the

opposed patent, and the application underlying it utterly fail to provide a sufficient basis for the reformulation of the technical problem attempted in the present case.

77. The problem initially suggested was the provision of a treatment for patients suffering from PNH using eculizumab:

> *"In certain aspects, the application provides a method of treating a patient suffering from paroxysmal nocturnal hemoglobinuria by administering a pharmaceutical composition comprising an antibody that binds C5 or an active antibody fragment thereof. In certain embodiments, the antibody that binds C5 or an active antibody fragment thereof is eculizumab."* (**WO**, p. li. 24 to 28)

This is supported by various similar statements throughout the original disclosure. In accordance with this interpretation, the Proprietor formulated the underlying problem of the original filing to lie in the provision of a useful treatment for patients suffering from PNH (see also again section 5.10.2 of the submission).

78. The Proprietor then alleges that the application as filed would justify reformulating the technical problem as the *"provision of a composition of eculizumab which demonstrates the ideal balance of properties for use in the treatment of PNH, including enhanced stability and thus maintenance of biological integrity following prolonged storage in the absence of preservative, whilst also providing a low-volume liquid formulation having acceptable infusion times and which is convenient to store"* (section 5.11.1 of the submission).

79. We note that this is not only a wholly artificial but also a highly specific problem, and one that is simply not derivable from the application as filed. Even if, as in the example given in the Guidelines at G-VII-11, pharmaceutical activity and toxicity were to be considered sufficiently related to allow for changing the technical problem accordingly, this is not so in the case of pharmaceutical activity and, e.g., enhanced stability, maintenance of biological integrity following prolonged storage in the absence of preservative, low-volume liquid formulation having acceptable infusion times and convenience to store, **let alone the provision of the allegedly "ideal combination" of all of the above** (which would obviously require some comparison to the prior art). Rather, these are galenic questions. The opposed patent is, however, not concerned with galenic questions at all.

80. In this regard, we refer to the Case Law Book which notes at page 458 of the English version (under item II.E.1.14) that reformulating the technical problem is not in breach of Art. 123(2) EPC provided the problem as clarified and the solution proposed could have been deduced from the application as a whole. In this regard, the Case Law Book cites **T 871/08** which

rejected the reformulation of the technical problem vis-à-vis the prior art requested in that case as follows:

> *4.3.4 … The board observes that a reformulation of the problem taking into account the above arguments would only be allowable, if the new problem could be deduced from the application as filed when considered in the light of the closest state in the art (see in this respect T 0013/84, OJ EPO 1986, 253; T 0530/90, point 4.3 of the reasons). In the present case, there is no basis in the application as filed for the provision of the so-called "safety margin for stable operation" that the respondent emphasised during the oral proceedings, nor can the latter be deduced from the application as filed by a person skilled in the art.*
>
> *4.3.5 It follows that the problem is to be reformulated in less ambitious terms, namely, to provide an alternative process for the production of hydrogen peroxide according to the anthraquinone process."* (Emphasis added)

Similarly to the concept of the "*safety margin for stable operation"* which the proprietor in **T 871/08** creatively (but eventually unsuccessfully) invented, there is no basis in the application as filed in the light of the closest prior art for reformulating the technical problem to the convoluted construct proposed by the Proprietor.

81. Hence the Proprietor's artificial and arbitrarily defined "ideal balance of properties" must not be used as basis for the discussion of inventive step already for legal reasons.

82. In any case, the data comprised in the original filing, but also in **D33**, by no means show that the claimed composition provides an ideal balance of properties. It is not quite clear what properties the Proprietor has in mind when making this bold claim, but undoubtedly one property that would appear to be highly relevant for a patient suffering from PNH is the biologic activity / drug activity of the active agent (cf. e.g. sections 4.3.1, 4.6.9, 5.3.1, 5.3.3 of the submission). In section 5.3.3 of the submission, the Proprietor states that

> *"[i]t is, of course, critical that the antibody maintains its biological integrity and activity in the period between manufacture and use."* (emphasis added)

83. However, neither the application as filed, nor **D33** contain any information on the activity of the antibody in the tested solutions. No comprehensive data on biological integrity is provided, either. As the Proprietor stated (section 4.3.3 of the submission) and as further discussed below, biological integrity depends on numerous different degradation pathways one of which is aggregation. The mere fact that an antibody does not aggregate over a certain period of time is by no means sufficient to prove that it actually retained its activity, let alone that a

solution exhibits an *"ideal balance of properties"*. Not even biological integrity can be reliably evaluated by this single parameter.

84. According to **D20** (p. 8 to 14), the major degradation pathways for antibodies are physical degradation (i.e., denaturation and aggregation) and chemical degradation (i.e., disulfide bond formation/exchange, nonreducible cross-linking, deamidation, isomerization, oxidation, formation of acidic or basic species, C-terminal clipping, fragmentation and/or Maillard reaction). Of all these pathways, the Proprietor only analyzed one, namely aggregation.

85. **D21** notes in this regard that

> *"Probably the most important aspect of identifying a successful antibody drug formulation is in the identification of truly detrimental alterations rather than all of the modifications that might occur. Although, from a regulatory standpoint, it may be beneficial to understand all of the potential degradation events associated with an antibody formulation, it is the recognition of a stability-indicating parameter (or several parameters) that allows the formulation scientist to focus in on what is critical to development of a successful product. Here, a series of physico-chemical and bioanalytical assays can be used to identify these parameters [32]. Although in vivo bioassays may ultimately be required to validate a stability-indicating formulation parameter, the most critical tools at the disposal of the formulation scientist are rapid and reproducible assays to measure protein changes due to oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification that could lead to a loss of function of the antibody drug."* (**D21**, p. 690, right column, 1st full paragraph, emphasis added)

86. In the present case, the proprietor picked one out of many aspects and chose to present it as being indicative of an *"ideal balance of properties"*. This is not credible by any means when judged in view of the prior art, or in view of the Proprietor's own statements. In fact, it is not even clear if aggregation belongs to the *"truly detrimental alterations"* described in **D21**, or if it is of minor significance for the specific antibody in question, eculizumab.

87. To evaluate any *"balance of properties"*, it would obviously be necessary to evaluate more than one property. At the very least it would appear appropriate to determine *"oxidation, deamidation, aggregation, fragmentation and other forms of chemical modification"* (**D21**, see above). One analysis that may be used to deliver a more complete picture is the rCE-SDS assay performed by Dr. Treuheit (**D23**, Annex C). It shows that there is no significant difference between the tested concentrations. If anything, it shows that 5 mg/mL is slightly less stable than the other tested samples. It does, however, not show any advantage in terms of overall stability (physical and chemical degradation) for the concentration of 10 mg/mL.

88. In summary, it is not credible from the application as filed, or the subsequently filed data (**D33**) that the technical problem as formulated by the Proprietor ("*provision of a composition of eculizumab which demonstrates the ideal balance of properties for use in the treatment of PNH, including enhanced stability and thus maintenance of biological integrity following prolonged storage […]*") has been solved. The original disclosure contains no data in this regard at all and **D33** does not in fact provide evidence for the alleged maintenance of biological integrity, but at best a minute effect, if any, on aggregation, which does not seem to be the most relevant variable for the maintenance of the biological integrity of eculizumab. Any assertion about "ideal" properties is mere speculation.

89. Hence, the technical problem put forward by the Proprietor cannot be derived from (or is insufficiently related to) to original disclosure and in any event has not been credibly solved. Thus, it cannot be accepted as basis for the evaluation of inventive step.

IV.4 The problem has in any event not been credibly solved over the whole scope claimed

90. Even if a concentration of 10 mg/mL was indeed accepted to provide any benefit over other concentrations in the specific formulation that was tested, and even if it were assumed that finding this solution would require an inventive effort (which it should not be for the above reasons), the technical problem would not be credibly solved over the whole scope claimed.

91. The Proprietor alleged in great detail that finding a suitable concentration for eculizumab was a highly challenging endeavor. While we disagree with this assessment, we note that if all of the Proprietor's arguments were indeed accepted as true, one would have to conclude that numerous relevant and in fact essential features are missing from the claim as granted.

92. The Proprietor argued that *"it is very important to maintain the integrity of the antibody and to minimize any possible degradation pathways"* (section 4.3.1 of the submission, emphasis added). Such degradation pathways would include *"denaturation, aggregation, surface adsorption, disulphide bond formation/exchange, non-reducible cross-linking, deamidation, isomerisation, oxidation, formation of acidic or basic species, C-terminal clipping, fragmentation and Maillard reaction. The skilled person looking to formulate an antibody therefore has a multitude of different problems to consider, each of which will apply in different ways to each individual antibody."* (Section 4.3.3 of the submission). As noted before, the data provided by the proprietor relates to only one of these pathways, namely aggregation.

93. The Proprietor further states that

> *"[…] the strategies available to minimize the various degradation pathways may conflict with one another. For instance, deamidation, a major degradation pathway in*

> *antibodies, has been shown to have a strong pH-dependence, with less deamidation occurring at pH 4 compared to pH 7 (**D20**, page 12, right-hand column penultimate paragraph). In contrast, antibody precipitation can be a significant problem at low pH values. **D20** reports (page 11, left-hand column first paragraph) that incubation of two mouse monoclonal antibodies (lgG2a and lgG 1) at 37° C for 32 days caused precipitation at pH 3 and pH 4 solutions for both antibodies. It is therefore difficult to create a stable antibody formulation due to the numerous degradation pathways possible, and the potentially conflicting strategies available to the formulation chemist for dealing with each of them in the context of each individual antibody."* (Section 4.3.5 of the submission)

94. Thus, if the Proprietor's statements are accepted, then optimization of the pH value of an antibody formulation is absolutely crucial to provide long term stability. Due to contrary tendencies with regard to different degradation pathways (deamidation and precipitation), such optimization is allegedly a challenging task. In any case, varying pH values will have a significant impact on the stability of the antibody in solution.

95. Another critical variable appears to be the buffer of the solution, according to the passage from **D21**, recited by the Proprietor in section 4.3.6:

> *"One approach is to find a pH where the minimal amount of degradation events such as deamidation and oxidation will occur. Another is to identify a buffer system that can stabilize the formulation at that pH without damaging the antibody."* (**D21**, p. 701, right hand column, emphasis added)

96. Thus, certain buffer systems may damage an antibody in solution, according to the Proprietor's submission, citing **D21**.

97. Further, specific salt concentrations may lead to dimerization of one particular antibody while not leading to aggregation of a closely related antibody (section 4.4.2 of the submission).

98. The fact that many stress factors may influence aggregation of an antibody during *inter alia* formulation and long term storage is summarized in **D34**, which was submitted by the Proprietor:

> *"Throughout these steps the antibody molecule may encounter several stress factors that can dramatically increase its propensity to aggregate (including variations of temperature, pH, protein concentrations, ionic strength, exposure to air-water interfaces and mechanical stress)."* (**D34**, p. 270, left column, 1st paragraph, emphasis added)

99. Thus, according to the Proprietor and the cited prior art, an individual antibody will be stable only at a specific concentration, at a specific pH, in a specific buffer with a specific amount of certain salts being present. In order to *"determine a suitable composition for the uniquely-engineered eculizumab antibody, the skilled person would have to conduct a significant research program to fully investigate the stability and biological integrity of the antibody, as well as the various factors (and their interplay) affecting each"* (section 5.5.5 of the submission, emphasis added).

100. However, of all the various factors, only the concentration is defined in the granted claim. Consequently, the alleged technical effect must solely be provided by the claimed concentration, at any pH, in any buffer, using any concentration of any salt, etc.

101. These parameters (including concentration) may in our view be determined by means of routine experimentation, particularly when starting from such a promising springboard as **D1** in combination with **D22** or **D42**. If, however, the OD was inclined to follow the Proprietor's argument that the identification of a suitable concentration requires inventive activity, this would equally apply to the other essential variables, such as buffer, pH and salt concentration of the antibody composition. In this case it would in fact appear that the vast majority of embodiments falling under the ambit of the claims could not possibly provide the claimed technical effect and thus could not be assumed to solve the problem, while at the same time the identification of functional embodiments would require an undue burden.

102. The Boards of Appeal have clearly decided, in particular in cases where the Applicant/Proprietor tries to reformulate the technical problem to be solved based on a subsequently invoked technical effect, that this can only be accepted if the problem was credibly/plausibly solved over the whole ambit of the claims. Further, the burden of proof in such a case lies with the Proprietor (see e.g. **T 1188/00**, point 4.5 of the reasons, **T 1417/05**, point 9.4 of the reasons, **T 1034/14**, point 5.4 of the reasons, **T 235/04** point 4.6 to 4.8 of the reasons). However, the Proprietor utterly failed to provide any evidence that the alleged technical effect is in fact provided over the whole scope of the claims.

103. As it is by no means credible/plausible that the technical effect of superior, ideal or merely suitable antibody stability over long periods of storage is provided by the concentration of 10 mg/mL over the whole range of conceivable pH values, buffers and salt concentrations, in particular not in light of the Proprietor's own arguments and the documents on file, the alleged technical effect must be disregarded for the assessment of inventive step (**T 668/94** point 8.3 of the reasons). Any other approach would be in stark contrast to the long standing principle that the extent of a patent monopoly, as defined by the claims, should reflect the technical contribution to the art (e.g. **T 939/92**, point 2.4.2 of the reasons, together with **T 409/91**, point 3.3 and 3.4 of the reasons).

104. The claim as granted lacks an inventive step at least because the technical problem has not been credibly / plausibly solved over the whole ambit of the claims, and for the reasons detailed above.

V. No Priority Entitlement (Article 87 EPC)

105. We have explained in our Notice of Opposition why the opposed patent is not entitled to the claimed priority of US 783070 P. We note that the Proprietor has not submitted any arguments regarding this issue. The filing date of the opposed patent, i.e. March 15, 2007 is thus considered to be the relevant date for any consideration of prior art.

VI. Summary

106. In view of all of the above, revocation of the patent in its entirety is fully justified.

Respectfully,

Dr. H. Ulrich Dörries
European Patent Attorney

UD/SI/KS

Enclosures
Documents **D39** to **D46**

D44

RITUXAN™
Rituximab

DESCRIPTION

The RITUXAN (Rituximab) antibody is a genetically engineered chimeric murine/human monoclonal antibody directed against the CD20 antigen found on the surface of normal and malignant B lymphocytes. The antibody is an IgG_1 kappa immunoglobulin containing murine light- and heavy-chain variable region sequences and human constant region sequences. Rituximab is composed of two heavy chains of 451 amino acids and two light chains of 213 amino acids (based on cDNA analysis) and has an approximate molecular weight of 145 kD. Rituximab has a binding affinity for the CD20 antigen of approximately 8.0 nM.

The chimeric anti-CD20 antibody is produced by mammalian cell (Chinese Hamster ovary) suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product. The anti-CD20 antibody is purified by affinity and ion exchange chromatography. The purification process includes specific viral inactivation and removal procedures.

RITUXAN is a sterile, clear, colorless, preservative-free liquid concentrate for intravenous (IV) administration. RITUXAN is supplied at a concentration of 10 mg/mL in either 100 mg (10 mL) or 500 mg (50 mL) single-use vials. The product is formulated for intravenous administration in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80, and Sterile Water for Injection. The pH is adjusted to 6.5.

CLINICAL PHARMACOLOGY

General

Rituximab binds specifically to the antigen CD20 (human B-lymphocyte-restricted differentiation antigen, Bp35), a hydrophobic transmembrane protein with a molecular weight of approximately 35 kD located on pre-B and mature B lymphocytes.[1,2] The antigen is also expressed on >90% of B-cell non-Hodgkin's lymphomas (NHL)[3] but is not found on hematopoietic stem cells, pro-B cells, normal plasma cells or other normal tissues.[4] CD20 regulates an early step(s) in the activation process for cell cycle initiation and differentiation,[4] and possibly functions as a calcium ion channel.[5] CD20 is not shed from the cell surface and does not internalize upon antibody binding.[6] Free CD20 antigen is not found in the circulation.[2]

Pre-clinical Pharmacology and Toxicology

Mechanism of Action: The Fab domain of Rituximab binds to the CD20 antigen on B-lymphocytes and the Fc domain recruits immune effector functions to mediate B-cell lysis *in vitro*. Possible mechanisms of cell lysis include complement-dependent cytotoxicity (CDC)[7] and antibody-dependent cellular cytotoxicity (ADCC). The antibody has been shown to induce apoptosis in the DHL-4 human B-cell lymphoma line.[8]

Normal Tissue Cross-reactivity: Rituximab binding was observed on lymphoid cells in the thymus, the white pulp of the spleen, and a majority of B-lymphocytes in peripheral blood and lymph nodes. Little or no binding was observed in non-lymphoid tissues examined.

Human Pharmacokinetics/Pharmacodynamics

In patients given single doses at 10, 50, 100, 250 or 500 mg/m² as an IV infusion, serum levels and the half-life of Rituximab were proportional to dose. In 9 patients given 375 mg/m² as an IV infusion for four doses, the mean serum half-life was 59.8 hours (range 11.1 to 104.6 hours) after the first infusion and 174 hours (range 26 to 442 hours) after the fourth infusion. The wide range of half-lives may reflect the variable tumor burden among patients and the changes in CD20 positive (normal and malignant) B-cell populations upon repeated administrations.

Rituximab at a dose of 375 mg/m² was administered as an IV infusion at weekly intervals for four doses to 166 patients. The peak and trough serum levels of Rituximab were inversely correlated with baseline values for the number of circulating CD20 positive B cells and measures of disease burden. Median steady-state serum levels were higher for responders compared to nonresponders; however, no difference was found in the rate of elimination as measured by serum half-life. Serum levels were higher in patients with International Working Formulation (IWF) subtypes B, C, and D as compared to those with subtype A. Rituximab was detectable in the serum of patients three to six months after completion of treatment.

The pharmacokinetic profile of Rituximab when administered as six infusions of 375 mg/m² in combination with six cycles of CHOP chemotherapy was similar to that seen with Rituximab alone.

Administration of RITUXAN resulted in a rapid and sustained depletion of circulating and tissue-based B cells. Lymph node biopsies performed 14 days after therapy showed a decrease in the percentage of B-cells in seven of eight patients who had received single doses of Rituximab ≥100 mg/m². [9] Among the 166 patients in the pivotal study, circulating B-cells (measured as CD19+ cells) were depleted within the first three doses with sustained depletion for up to 6 to 9 months post-treatment in 83% of patients. One of the responding patients (1%), failed to show significant depletion of CD19+ cells after the third infusion of Rituximab as compared to 19% of the nonresponding patients. B-cell recovery began at approximately six months following completion of treatment. Median B-cell levels returned to normal by twelve months following completion of treatment.

There were sustained and statistically significant reductions in both IgM and IgG serum levels observed from 5 through 11 months following Rituximab administration. However, only 14% of patients had reductions in IgG and/or IgM serum levels, resulting in values below the normal range.

CLINICAL STUDIES

A multicenter, open-label, single-arm study was conducted in 166 patients with relapsed or refractory low-grade or follicular B-cell NHL who received 375 mg/m² of RITUXAN given as an IV infusion weekly for four doses. Patients with tumor masses >10 cm or with >5,000 lymphocytes/µL in the peripheral blood were excluded from the study. The overall response rate (ORR) was 48% (80/166) with a 6% (10/166) complete response (CR) and a 42% (70/166) partial response (PR) rate. Disease-related signs and symptoms (including B-symptoms) were present in 23% (39/166) of patients at study entry and resolved in 64% (25/39) of those patients. The median time to onset of response was 50 days and the median duration of response is projected to be 10 to 12 months.

In a multivariate analysis, the ORR was higher in patients with IWF B, C, and D histologic subtypes as compared to IWF A subtype (58% vs. 12%), higher in patients whose largest lesion was <5 cm vs. >7 cm in greatest diameter (55% vs. 38%), and higher in patients with chemosensitive relapse as compared to chemoresistant (defined as duration of response <3 months) relapse (53% vs. 36%). ORR in patients previously treated with autologous bone marrow transplant was 78% (18/23). The following factors were not associated with a lower response rate: age ≥ 60 years, extranodal disease, prior anthracycline therapy, and bone marrow involvement.

In a second multicenter, multiple-dose study, 37 patients with relapsed or refractory B-cell NHL received 375 mg/m² of RITUXAN as an IV infusion once weekly for four doses.[10,11] The ORR was 46% with a median duration of response of 8.6 months (range 2.6 to 26.2+). Single doses of up to 500 mg/m² were well-tolerated.[9]

Twenty patients have received two courses and one patient has received three courses of RITUXAN as 4 weekly infusions of 375 mg/m² per infusion. The percentage of patients reporting adverse events upon retreatment was similar to that reported following the first course, although the incidence of specific adverse events differed (see ADVERSE EVENTS). All patients had obtained an objective clinical response (CR or PR) to the first course of RITUXAN™ (Rituximab); upon retreatment, 6 of 12 patients evaluable for response obtained a complete or partial remission.

Twenty-nine patients with relapsed or refractory, bulky (single lesion of >10 cm in diameter), low grade NHL received 375 mg/m² of RITUXAN as four weekly infusions. The overall incidence of adverse events and the incidence of Grade 3 and 4 adverse events was higher in patients with bulky disease than in patients with non-bulky disease (see ADVERSE EVENTS). Ten of 21 patients evaluable for response have obtained a complete or partial remission.

INDICATIONS AND USAGE

RITUXAN is indicated for the treatment of patients with relapsed or refractory low-grade or follicular, CD20 positive, B-cell non-Hodgkin's lymphoma.

CONTRAINDICATIONS

RITUXAN is contraindicated in patients with known Type I hypersensitivity or anaphylactic reactions to murine proteins or to any component of this product. (See WARNINGS.)

WARNINGS

RITUXAN is associated with hypersensitivity reactions which may respond to adjustments in the infusion rate. Hypotension, bronchospasm, and angioedema have occurred in association with RITUXAN infusion as part of an infusion-related symptom complex. RITUXAN infusion should be interrupted for severe reactions and can be resumed at a 50% reduction in rate (e.g., from 100 mg/hr to 50 mg/hr) when symptoms have completely resolved. Treatment of these symptoms with diphenhydramine and acetaminophen is recommended; additional treatment with bronchodilators or IV saline may be indicated. In most cases, patients who have experienced non-life-threatening reactions have been able to complete the full course of therapy. (See DOSAGE and ADMINISTRATION.) Medications for the treatment of hypersensitivity reactions, e.g., epinephrine, antihistamines and corticosteroids should be available for immediate use in the event of a reaction during administration.

Infusions should be discontinued in the event of serious or life-threatening cardiac arrhythmias. Patients who develop clinically significant arrhythmias should undergo cardiac monitoring during and after subsequent infusions of RITUXAN. Patients with preexisting cardiac conditions including arrhythmias and angina have had recurrences of these events during RITUXAN therapy and should be monitored throughout the infusion and immediate post-infusion period.

PRECAUTIONS

Laboratory Monitoring: Complete blood counts (CBC) and platelet counts should be obtained at regular intervals during RITUXAN therapy and more frequently in patients who develop cytopenias (see ADVERSE EVENTS).

Drug/Laboratory Interactions: There have been no formal drug interaction studies performed with RITUXAN.

HAMA/HACA Formation: Human anti-murine antibody (HAMA) was not detected in 67 patients evaluated. Less than 1.0% (3/355) of patients evaluated for human anti-chimeric antibody (HACA) were positive. Patients who develop HAMA/HACA titers may have allergic or hypersensitivity reactions when treated with this or other murine or chimeric monoclonal antibodies.

Immunization: The safety of immunization with any vaccine, particularly live viral vaccines, following RITUXAN therapy has not been studied. The ability to generate a primary or anamnestic humoral response to any vaccine has also not been studied.

Carcinogenesis, Mutagenesis, Impairment of Fertility: No long-term animal studies have been performed to establish the carcinogenic or mutagenic potential of RITUXAN, or to determine its effects on fertility in males or females. Individuals of childbearing potential should use effective contraceptive methods during treatment and for up to 12 months following RITUXAN therapy.

Pregnancy Category C: Animal reproduction studies have not been conducted with RITUXAN. It is not known whether RITUXAN can cause fetal harm when administered to a pregnant woman or whether it can affect reproductive capacity. Human IgG is known to pass the placental barrier, and thus may potentially cause fetal B-cell depletion; therefore, RITUXAN should be given to a pregnant woman only if clearly needed.

Nursing Mothers: It is not known whether RITUXAN is excreted in human milk. Because human IgG is excreted in human milk and the potential for absorption and immunosuppression in the infant is unknown, women should be advised to discontinue nursing until circulating drug levels are no longer detectable. (See CLINICAL PHARMACOLOGY.)

Pediatric Use: The safety and effectiveness of RITUXAN in children have not been established.

ADVERSE REACTIONS

Safety data are based on 315 patients treated in five single-agent studies of RITUXAN. This includes patients with bulky disease (lesions >10 cm), those who have received more than one course of RITUXAN, and patients receiving 375 mg/m² for eight doses.

Infusion-Related Events: An infusion-related symptom complex consisting of fever and chills/rigors occurred in the majority of patients during the first RITUXAN infusion. Other frequent infusion-related symptoms included nausea, urticaria, fatigue, headache, pruritus, bronchospasm, dyspnea, sensation of tongue or throat swelling (angioedema), rhinitis, vomiting, hypotension, flushing, and pain at disease sites. These reactions generally occurred within 30 minutes to 2 hours of beginning the first infusion, and resolved with slowing or interruption of the RITUXAN infusion and with supportive care (IV saline, diphenhydramine, and acetaminophen). The incidence of infusion-related events decreased from 80% (7% Grade 3/4) during the first infusion to approximately 40% (5% to 10% Grade 3/4) with subsequent infusions. Mild to moderate hypotension requiring interruption of RITUXAN infusion with or without the administration of IV saline occurred in 32 (10%) patients. Isolated occurrences of severe reactions requiring epinephrine have been reported in patients receiving RITUXAN for other indications. Angioedema was reported in 41 (13%) patients and was serious in one patient. Bronchospasm occurred in 25 (8%) patients; one-quarter of these patients were treated with bronchodilators. A single report of bronchiolitis obliterans was noted.

Immunologic Events: RITUXAN induced B-cell depletion in 70 to 80% of patients and was associated with decreased serum immunoglobulins in a minority of patients. The incidence of infection does not appear to be increased. During the treatment period, 50 patients in the pivotal trial developed 68 infectious events; 6 (9%) were Grade 3 in severity and none were Grade 4 events. Of the

Samsung Bioepis Exhibit No. 1002
Page 7871

6 serious infectious events, none were associated with neutropenia. The serious bacterial events included sepsis due to Listeria (n=1), Staphylococcal bacteremia (n=1) and polymicrobial sepsis (n=1). In the post-treatment period (30 days to 11 months following the last dose), bacterial infections included sepsis (n=1); significant viral infections included herpes simplex infections (n=2) and herpes zoster (n=3).

Retreatment Events: Twenty-one patients have received more than one course of RITUXAN™ (Rituximab). The percentage of patients reporting any adverse event upon retreatment was similar to the percentage of patients reporting adverse events upon initial exposure. The following adverse events were reported more frequently in retreated subjects: asthenia, throat irritation, flushing, tachycardia, anorexia, leukopenia, thrombocytopenia, anemia, peripheral edema, dizziness, depression, respiratory symptoms, night sweats, and pruritus.

Hematologic Events: During the treatment period (up to 30 days following last dose) severe thrombocytopenia occurred in 1.3% of patients, severe neutropenia occurred in 1.9% of patients, and severe anemia occurred in 1.0% of patients. A single occurrence of transient aplastic anemia (pure red cell aplasia) and two occurrences of hemolytic anemia following RITUXAN therapy were reported.

Cardiac Events: Four patients developed arrhythmias during RITUXAN infusion. One of the four discontinued treatment because of ventricular tachycardia and supraventricular tachycardias. The other three patients experienced trigeminy (1) and irregular pulse (2) and did not require discontinuation of therapy. Angina was reported during infusion and myocardial infarction occurred 4 days post-infusion in one subject with a prior history of myocardial infarction.

Table 1.

Adverse Events ≥5% of Patients (N=315)

	Incidence All Grades	
	N	%
Any Adverse Event	**275**	**87**
Body As A Whole		
Fever	154	49
Chills	102	32
Asthenia	49	16
Headache	43	14
Throat Irritation	19	6
Abdominal Pain	18	6
Cardiovascular System		
Hypotension	32	10
Digestive System		
Nausea	55	18
Vomiting	23	7
Hemic and Lymphatic System		
Leukopenia	33	11
Thrombocytopenia	25	8
Neutropenia	21	7
Metabolic and Nutritional System		
Angioedema	41	13
Musculo-Skeletal System		
Myalgia	21	7
Nervous System		
Dizziness	23	7
Respiratory System		
Rhinitis	25	8
Bronchospasm	24	8
Skin and Appendages		
Pruritus	32	10
Rash	31	10
Urticaria	24	8

Severe and life-threatening (Grade 3 and 4) events were reported in 10% (32/315) of patients. The following Grade 3 and 4 adverse events were reported: neutropenia (1.9%), chills (1.6%), leukopenia and thrombocytopenia (1.3% for each), hypotension, anemia, bronchospasm, and urticaria (1.0% for each), headache, abdominal pain, arrhythmia (0.6% for each), and asthenia, hypertension, nausea, vomiting, coagulation disorder, angioedema, arthralgia, pain, rhinitis, increased cough, dyspnea, bronchiolitis obliterans, hypoxia, asthma, pruritus, and rash (one patient each, 0.3%).

The following adverse events occurred in ≥1.0% but <5.0% of patients, in order of decreasing incidence: flushing, artbralgia, diarrhea, anemia, cough increase, hypertension, lacrimation disorder, pain, hyperglycemia, back pain, peripheral edema, paresthesia, dyspepsia, chest pain, anorexia, anxiety, malaise, tachycardia, agitation, insomnia, sinusitis, conjunctivitis, abdominal enlargement, postural hypotension, LDH increase, hypocalcemia, hypesthesia, respiratory disorder, tumor pain, pain at injection site, bradycardia, hypertonia, nervousness, bronchitis, and taste perversion.

The proportion of patients reporting any adverse event was similar in patients with bulky disease and those with lesions <10 cm in diameter. However, the incidence of dizziness, neutropenia, thrombocytopenia, myalgia, anemia and chest pain was higher in patients with lesions >10 cm. The incidence of any Grade 3 and 4 event was higher (31% vs. 13%) and the incidence of Grade 3 or 4 neutropenia, anemia, hypotension, and dyspnea was also higher in patients with bulky disease compared with patients with lesions <10 cm.

OVERDOSAGE

There has been no experience with overdosage in human clinical trials. Single doses higher than 500 mg/m² have not been tested.

DOSAGE AND ADMINISTRATION

Usual Dose:

The recommended dosage of RITUXAN is 375 mg/m² given as an IV infusion once weekly for four doses (days 1, 8, 15, and 22). RITUXAN may be administered in an outpatient setting. **DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. (See Administration.)**

Instructions for Administration

Preparation for Administration: Use appropriate aseptic technique. Withdraw the necessary amount of RITUXAN and dilute to a final concentration of 1 to 4 mg/mL into an infusion bag containing either 0.9% Sodium Chloride USP or 5% Dextrose in Water USP. Gently invert the bag to mix the solution. Discard any unused portion left in the vial. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration.

RITUXAN solutions for infusion are stable at 2° to 8°C (36° to 46°F) for 24 hours and at room temperature for an additional 12 hours. No incompatibilities between RITUXAN and polyvinylchloride or polyethylene bags have been observed.

Administration: DO NOT ADMINISTER AS AN INTRAVENOUS PUSH OR BOLUS. Hypersensitivity reactions may occur (see WARNINGS). Premedication, consisting of acetaminophen and diphenhydramine, should be considered before each infusion of RITUXAN™ (Rituximab). Premedication may attenuate infusion-related events. Since transient hypotension may occur during RITUXAN infusion, consideration should be given to withholding antihypertensive medications 12 hours prior to RITUXAN infusion.

First Infusion: The RITUXAN solution for infusion should be administered intravenously at an initial rate of 50 mg/hr. RITUXAN should not be mixed or diluted with other drugs. If hypersensitivity or infusion-related events do not occur, escalate the infusion rate in 50 mg/hr increments every 30 minutes, to a maximum of 400 mg/hr. If hypersensitivity or an infusion-related event develops, the infusion should be temporarily slowed or interrupted (see WARNINGS). The infusion can continue at one-half the previous rate upon improvement of patient symptoms.

Subsequent Infusions: Subsequent RITUXAN infusions can be administered at an initial rate of 100 mg/hr, and increased by 100 mg/hr increments at 30-minute intervals, to a maximum of 400 mg/hr as tolerated.

Stability and Storage: RITUXAN vials are stable at 2° to 8°C (36° to 46°F). Do not use beyond expiration date stamped on carton. RITUXAN vials should be protected from direct sunlight.

HOW SUPPLIED

RITUXAN is supplied as 100 mg and 500 mg of sterile, preservative-free, single-use vials.
Single unit 100 mg carton: Contains one 10 mL vial of RITUXAN (10 mg/mL).
NDC 50242-051-21
Single unit 500 mg carton: Contains one 50 mL vial of RITUXAN (10 mg/mL).
NDC 50242-053-06

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Samsung Bioepis Exhibit No. 1002
Page 7872

Opposition against EP 2 359 834 B1
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Declaration by Dr. Ronald Taylor

I, Ronald Taylor, Ph.D., declare that:

1. I was employed as a Professor of Biochemistry and Molecular Genetics at the University of Virginia from 1973 to 2016. I retired in 2016 and I am now Professor Emeritus and I still have an active work schedule based on submitting papers for publication, giving invited talks, serving on a study section, and reviewing papers. For almost 30 years, my laboratory has had a productive record in the study and characterization of monoclonal antibodies (mAbs), including humanized mAbs, and their use in clinical applications. A major focus of my research is anti-CD20 mAbs. In collaboration with investigators at the NIH and the Mayo Clinic, we have participated in numerous pilot clinical trials testing the feasibility of changing amount and frequency of dosing for CD20 mAb therapies for CLL. My work has resulted in 199 publications in peer-reviewed journals. A current copy of my CV is provided as **Appendix A.**

2. I have reviewed U.S. Patent Application Publication No. 2005/0191298 ("**D1**"), which is titled "Method of Treating Hemolytic Disease" and published on September 1, 2005. **D1** discloses that patients with paroxysmal nocturnal hemoglobinuria (PNH) were treated by administering eculizumab in four weekly 600 mg intravenous infusions, followed one week later by a dose of 900 mg, and then 900 mg on a bi-weekly basis (**D1**, [0082]). **D1** further discloses that anti-C5 mAbs such as eculizumab "can be administered in a variety of unit dosage forms" (**D1**, [0058]-[0060], [0062]).

3. The skilled artisan would have understood that pharmaceutical unit dosage forms containing a mAb should not be shared among patients or returned to storage after opening, in order to prevent contamination of the dosage form and any unnecessary health risks to the patient being treated with the dosage form. The skilled artisan also would have understood that

Samsung Bioepis Exhibit No. 1002
Page 7873

wasting mAb increases medical costs to the patient without any commensurate benefit. Thus, the skilled artisan would have had reason to prepare a single-use dosage form of eculizumab that could be used to administer both of the doses disclosed by **D1** (600 mg and 900 mg) without leaving any leftover drug as unusable waste. The skilled artisan would have understood that the most efficient way to meet that goal would be to prepare a 300 mg single-use dosage form because that dosage form could be used to administer both of the doses disclosed by **D1**—*i.e.*, two, 300 mg single-use dosage forms to administer the 600 mg dose and three, 300 mg single-use dosage forms to administer the 900 mg dose. Therefore, a 300 mg single-use dosage form would have been the most obvious and convenient single-use dosage form for preparing both the 600 mg and 900 mg doses of eculizumab for treating PNH.

4. The skilled artisan also would have recognized that preparing a single dosage form that could flexibly be used to administer either 600 mg or 900 mg offered advantages over developing a separate dosage form for each of the 600 mg and 900 mg doses. Developing any given dosage form requires testing, validating, manufacturing, storing, and transporting that dosage form. The skilled artisan would have known that it would be more efficient to avoid the redundancy and simply find a dosage form that could be used to administer both desired doses rather than creating multiple different dosage forms that would each require individual testing and validation, and may each require different storage or transport set-ups. Accordingly, efficiency would have provided the skilled artisan with further reason to use a 300 mg single-use dosage form.

5. One example of aligning antibody dosage form and administered doses known as of 2006 is the administration of the therapeutic antibody rituximab. Rituximab was administered in doses of 500 or 1000 mg to treat rheumatoid arthritis (e.g., Emery *et al.*, *Arthritis & Rheumatism* 2006, *54(5)*, 1390-1400 ("**D43**") at p. 1390). Consistent with maximizing efficiency and minimizing waste, the dosage forms of rituximab available as of 2006 were supplied as 100 mg and 500 mg sterile, preservative-free, single-use vials—both of which could be used to administer either a 500 or 1000 mg dose without waste and with maximal efficiency (e.g., Rituximab 1997 Product Data Sheet ("**D44**") at p. 2, right column, "How Supplied" section).

Samsung Bioepis Exhibit No. 1002
Page 7874

6. In preparing the 300 mg eculizumab single-use dosage form, the skilled artisan also would have considered the appropriate concentration at which to formulate the mAb. Eculizumab is a humanized mAb with a hybrid IgG2/IgG4 constant domain. U.S. Patent No. 6,407,213 ("**D42**") is titled "Method for Making Humanized Antibodies" and issued on June 18, 2002. **D42** relates to methods for making and using humanized antibodies, including antibodies with hybrid constant domains (**D42**, col. 8, ll. 47-51). **D42** states that such humanized "antibodies will typically be formulated … at concentrations of about 1 mg/ml to 10 mg/ml" (**D42**, col. 45, li. 57, to col. 46, li. 2). Thus, selection of 10 mg/ml as a concentration for a humanized mAb with a hybrid IgG2/IgG4 constant domain, such as eculizumab, would have been a typical, arbitrary choice among known concentrations. Similarly, U.S. Patent Application Publication No. 2005/0271660 ("**D22**") discloses using eculizumab at a concentration between 1 and 30 mg/ml in nebulized formulations (**D22**, Fig. 10, [0170]-[0172]). From this, the skilled artisan would have understood that eculizumab is sufficiently stable and biologically active to be used as a drug at concentrations including 10 mg/ml. Thus, **D22** reinforces that selection of 10 mg/ml as a concentration for eculizumab would have been a typical, arbitrary choice among known concentrations, regardless of the route of administration. **D44** reinforces the typical nature of a 10 mg/ml concentration because it discloses that rituximab was supplied at that concentration (e.g., **D44** at p. 2, right column, "How Supplied" section).

7. Additionally, in choosing among these *typical* concentrations, the skilled artisan would have had a reason to prepare the 300 mg single-use dosage form using a 10 mg/ml concentration of eculizumab in order to minimize the volume of the 600 mg and 900 mg doses required for treating PNH. For example, preparing the 600 mg and 900 mg doses taught by **D1** would require 60 ml and 90 ml, respectively, of a 300 mg single-use dosage form having 10 mg/ml of the mAb. But preparing the same 600 mg and 900 mg doses would require 600 ml and 900 ml, respectively, of a 300 mg single-use dosage form having 1 mg/ml of the antibody. The skilled artisan would have understood that smaller volumes would be more convenient for storage, transportation, and administration of the mAb. Further, the skilled artisan would have been reluctant to use concentrations higher than the 10 mg/ml taught by **D42** because increased aggregation was known to result from increased concentration of antibodies (e.g., Wang *et al*., *J. Pharm. Sci*. 2007, *96(1)*, 1-26 ("**D20**") at p. 9, left column, 2nd full paragraph).

Samsung Bioepis Exhibit No. 1002
Page 7875

8. Given that the skilled artisan would have had reason to use a 300 mg single-use dosage form with a concentration of 10 mg/mL, the skilled artisan necessarily would have arrived at a 30 mL volume. The volume of a 10 mg/mL solution needed to prepare 300 mg is, and can only be, 30 mL.

I certify that the above statements are correct to the best of my knowledge and belief.

Keswick, Virginia Nov 20, 2018
Place and Date

Dr. Ronald Taylor

Samsung Bioepis Exhibit No. 1002
Page 7876

Appendix A

Samsung Bioepis Exhibit No. 1002
Page 7877

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Ronald P. Taylor

eRA COMMONS USER NAME (credential, e.g., agency login): RPTNIH

POSITION TITLE: Emeritus Professor of Biochemistry and Molecular Genetics, University of Virginia Health Science Center

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
City College of New York, New York, NY	B.S.	1966	Chemistry
Princeton University, Princeton, NJ	Ph.D.	1970	Physical Chemistry
University of Minnesota, Mpls., MN	Post-doc	1971-1973	Physical Chemistry

A. Personal Statement

For almost 30 years my laboratory has had a long and productive record in the study and characterization of monoclonal antibodies and their use in several different clinical applications. We have a considerable level of experience in studying complement activation by, as well as effector cell-mediated killing of, tumor cells opsonized with monoclonal antibodies. Our laboratory discovered and has substantially characterized the shaving/trogocytosis reaction: that is, the loss of CD20 that occurs when CLL patients are treated with the usual (high) doses of rituximab or of ofatumumab. We have demonstrated, in several pilot clinical studies as well as in vitro investigations that much lower doses of CD20 mAbs, given more frequently, may provide far more effective therapies for CLL. We have also now described, in some detail at the cellular level, the complex series of events that occur when mAb-opsonized nucleated cells are killed by complement. Our laboratory also first described the exhaustion of complement which follows high dose rituximab therapy in CLL treatment.

I was appointed Professor Emeritus as of July 1, 2016. Since then I have maintained on-going collaborations with investigators in the US: Dr. D. Portilla (UVA), Dr. C. Zent (Rochester), Dr. A. Wiestner (NIH), Dr. J. Lambris (Penn), Dr. J. Thurman (U Colorado), Dr. S. Ram (U Mass); and in Europe: Dr. T. Mollnes (Norway) Dr. M. Huber-Lang (Germany), Dr. A. Verschoor (Germany) and the Genmab Biotech Company (The Netherlands).

B. Positions and Honors

Professional Positions:

1973-1978, Assistant Professor, Dept. of Biochemistry, University of Virginia.
1978-1983, Associate Professor, Dept. of Biochemistry, University of Virginia.
1983-present, Professor, Dept. of Biochemistry and Molecular Genetics, University of Virginia.
1991-present, Member, Beirne B. Carter Center for Immunology Research, University of Virginia.
1997-present, Member, NCI Sponsored Cancer Center, University of Virginia.

Awards and Other Professional Activities:

1965, Phi Beta Kappa; 1966, ECAC Scholar-Athlete Award; 1966-1969, NDEA Graduate Fellow; 1970, NSF Graduate Fellow; 1972-1973, NIH Fellow; 1975-1980, NIH Research Career Development Award; 1992, Ad

Samsung Bioepis Exhibit No. 1002
Page 7878

Hoc Study Section, Immunological Sciences, NIH; 1991-1993, Shannon Award, NIH; 1990-1994, Editorial Board, Arthritis and Rheumatism; 1990, Fellowship Subcommittee, Arthritis Fdn; 2008, DARPA Significant Technical Achievement Award; 2010-2015, Editorial Board, Haematologica; 2010-2014, Associate Editor, Journal of Immunology; Ad hoc reviewer: J. Immunology, J. Immunological Methods, J. Clin Invest, Blood, Mol. Immunology, Immunology, Science Translational Medicine, and many Cancer Journals: Reviewer, NIH: 2003-2004, Exptl. Immunol; 2004,Tumor Immunol./Immunotherapy; 2007-2018 (ongoing), Bench to Bedside NIH Study Section.

C. Contributions to Science (total publications: 199)

1. Our laboratory has pioneered the detailed and comprehensive evaluation of blood samples taken from patients before, during, and after CD20 therapy for CLL. This work led to our discovery of the shaving/trogocytosis reaction, as well as to the finding that immune effector functions in the body, such as complement, can be saturated/exhausted when CLL patients receive the usual high CD20 mAb doses (300 mg to 2 grams). This work also directly led to our in vitro analyses and mechanistic descriptions of trogocytosis, in which we demonstrated that it is mediated by Fcγ receptors on effector cells including monocyte/macrophages, neutrophils, and NK cells. We have also described the process in a mouse model and demonstrated that the reaction can be generalized to include other FDA-approved immunotherapeutic mAbs including cetuximab and traustuzumab. Finally, in collaboration with investigators at the NIH and the Mayo Clinic, we have been able to participate in numerous pilot clinical trials that have indicated the potential feasibility of changing CD20 mAb therapies for CLL to low doses (~35 mg) three times a week, which ultimately can be based on subcutaneous infusions. If this approach can be clearly validated and generalized, it would provide a very important and revolutionary paradigm shift that could benefit both patients and health-care providers, based on efficacy, costs, and convenience.

Most recently, in collaboration with several investigators we have also been studying how complement plays a role in the pathogenesis of several diseases and syndromes associated with inflammation and tissue damage, including malaria and kidney injury due to tubulointerstitial fibrosis. This work has the potential to lead to new and innovative strategies for disease treatment, based on selective inhibition of key steps in the complement cascade(s).

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Samsung Bioepis Exhibit No. 1002
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2. A second focus in our laboratory has been based on studying the basic scientific details by which mAbs promote CDC of targeted cells. This led to our discovery of "streamers," or tunneling nanotubules, that are released when the complement membrane attack complex penetrates the cell and large amounts of calcium enter the cytoplasm. We have also been able to describe the sequence of cellular events that occurs during the CDC process, based on 4-color movies generated in confocal microscopy experiments. This work, in collaboration with the biotech company Genmab, also led to our description of hexamer-forming mAbs that are

Samsung Bioepis Exhibit No. 1002
Page 7880

far more effective than wild-type mAbs in promoting CDC. It is very likely that these next generation mAbs (for CD20 as well as other targets) will find clinical applications in the not-too-distant future.

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Samsung Bioepis Exhibit No. 1002
Page 7881

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Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti–DC-SIGN antibody

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Current dendritic cell (DC)–based vaccines are based on ex vivo–generated autologous DCs loaded with antigen prior to readministration into patients. A more direct and less laborious strategy is to target antigens to DCs in vivo via specific surface receptors. Therefore, we developed a humanized antibody, hD1V1G2/G4 (hD1), directed against the C-type lectin DC-specific intercellular adhesion molecule 3–grabbing nonintegrin (DC-SIGN) to explore its capacity to serve as a target receptor for vaccination purposes. hD1 was cross-linked to a model antigen, keyhole limpet hemocyanin (KLH). We observed that the chimeric antibody-protein complex (hD1-KLH) bound specifically to DC-SIGN and was rapidly internalized and translocated to the lysosomal compartment. To determine the targeting efficiency of hD1-KLH, monocyte-derived DCs and peripheral blood lymphocytes (PBLs) were obtained from patients who had previously been vaccinated with KLH-pulsed DCs. Autologous DCs pulsed with hD1-KLH induced proliferation of patient PBLs at a 100-fold lower concentration than KLH-pulsed DCs. In addition, hD1-KLH–targeted DCs induced proliferation of naive T cells recognizing KLH epitopes in the context of major histocompatibility complex (MHC) classes I and II. We conclude that antibody-mediated targeting of antigen to DCs via DC-SIGN effectively induces antigen-specific naive as well as recall T-cell responses. This identifies DC-SIGN as a promising target molecule for DC-based vaccination strategies. (Blood. 2005;106:1278-1285)

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Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play a key role in regulating antigen-specific immunity. DCs capture antigens, process them into peptides, and present these to T cells.[1] The interaction between DC and T-cell controls the type and magnitude of the resulting immune response. Recently, preclinical and clinical studies have exploited DCs in an attempt to improve vaccine efficacy.[2] Most of these studies involve ex vivo antigen loading of autologous monocyte-derived DCs that are readministrated to the patient, a laborious and costly procedure. A more direct strategy involves targeting of antigens specifically to antigen uptake receptors on the DC in vivo. Potential candidate receptors highly expressed by DCs include Fc receptors[3-5] and members of the C-type lectin family.[6,7] Whereas Fc receptors are expressed by many different cell types, the expression of some members of the C-type lectin family are more DC restricted.[8]

C-type lectins bind sugar residues in a calcium-dependent manner via a highly conserved carbohydrate recognition domain. C-type lectin receptors expressed by DCs are implicated in immunoregulatory processes, such as antigen capture, DC trafficking, and DC–T-cell interactions.[8] Based on the location of the amino (N) terminus, 2 types of membrane-bound C-type lectins can be distinguished on DCs. Type I C-type lectins have their N terminus located outside, while type II C-type lectins have their N terminus located inside the cell. Several studies have been conducted on antigen targeting to C-type lectin receptors for vaccination purposes, mainly focusing on the type I C-type lectins mannose receptor (MR)[9] and DEC-205.[6,10,11] Vaccines based on natural MR ligands have been shown to effectively induce humoral and cellular responses.[9] However, these ligands lack specificity for the MR, and may target multiple lectins with overlapping binding specificities, including soluble lectins and lectin receptors expressed by cells that are not specialized in antigen presentation. More specific receptor targeting can be obtained by the use of antibodies directed against specific C-type lectins, a strategy that has been successfully applied in MR[7,12] and DEC-205[6,10,11] targeting studies. Antibody-mediated targeting of antigen to the MR on human DCs results in antigen presentation and activation of naive T cells in vitro.[7,12] Moreover, in vivo studies on antibody-mediated targeting of DEC-205 in mice demonstrate presentation of the antigen to naive $CD4^+$ and $CD8^+$ T cells.[6,10]

DC-specific intercellular adhesion molecule 3 (ICAM-3)–grabbing nonintegrin (DC-SIGN) represents a member of the

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Samsung Bioepis Exhibit No. 1002
Page 7882

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type II C-type lectin family. We have previously demonstrated that DC-SIGN is an endocytic receptor mediating antigen presentation.[13] A major advantage of targeting DC-SIGN over other C-type lectin receptors is its expression pattern. In humans, DC-SIGN expression is restricted to professional APCs and expression levels are high. Human DC-SIGN is abundantly expressed by DCs residing in lymphoid tissues and at mucosal surfaces, dermal DCs, and by specialized macrophages in placenta and lung.[14,15]

Targeting constructs that are to be used in humans should consist of antibodies that do not elicit immune responses directed against the antibody itself. Recent developments in antibody engineering provide the tools for the production of either humanized or human antibodies.[16] Undesired interactions between the targeting antibody and Fc receptors can be avoided by use of single-chain Fv constructs or composite immunoglobulin G (IgG) molecules,[17] thus enhancing targeting specificity.

Here we evaluate the effectiveness of targeting antigen to human DCs via DC-SIGN. For targeting purposes, keyhole limpet hemocyanin (KLH), a large globular protein containing a large array of immunogenic epitopes, was chosen as a model antigen. KLH is widely used in clinical DC-based vaccination trials for immunomonitoring purposes, and is thought to stimulate cytotoxic T-cell responses by recruiting bystander T-cell help.[2] KLH was chemically cross-linked to a humanized anti–DC-SIGN IgG2/IgG4 composite antibody (hD1), resulting in the chimeric hD1-KLH protein. The results demonstrate that hD1-KLH was capable of inducing T-cell responses at a 100-fold lower concentration than KLH alone.

Patients, materials, and methods

Antibodies and reagents

The following antibodies were used: AZN-D1 (IgG1, mouse antihuman DC-SIGN),[14] AZN-L19 (IgG1, mouse antihuman CD18),[18] W6/32 (IgG2a, mouse anti–HLA-A, –HLA-B, –HLA-C; ATCC, Manassas, VA), IVA-12 (IgG1, mouse anti–HLA-DR, –HLA-DP, –HLA-DQ; ATCC), mouse IgG1 isotype (R&D Systems, Abingdon, United Kingdom), total mouse IgG (Jackson ImmunoResearch; Brunschwig Chemie B.V., Amsterdam, the Netherlands), Alexa Fluor 647–labeled goat anti–human IgG (Molecular Probes, Leiden, the Netherlands), and Alexa Fluor 647–labeled goat anti–mouse IgG1 (Molecular Probes). Endotoxin-free KLH was purchased from Calbiochem (La Jolla, CA).

Recombinant antibodies

The humanized antihuman DC-SIGN antibody hD1V1G2/G4 (hD1) was generated by complementarity determining region (CDR) grafting of AZN-D1 hypervariable domains into human framework regions. The humanized variable heavy and variable light regions were then genetically fused with a human hybrid IgG2/IgG4 constant domain[17] and a human kappa chain constant domain, respectively. This construct was cloned into a mammalian expression vector and the final construct transfected into NSO cells. Stable transfectants were obtained using glutamate synthetase (GS) selection (Lonza Biologics, Portsmouth, NH). Supernatants containing hD1 were purified over a protein A column. An isotype control antibody, h5G1.1-mAb (5G1.1, eculizamab; Alexion Pharmaceuticals) containing the same IgG2/IgG4 constant region, is specific for the human terminal complement protein C5.[19]

Generation of hD1-KLH

The chemical cross-linker sulfosuccinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sSMCC; Pierce, Rockford, IL) was conjugated to KLH according to the manufacturer's protocol. Protected sulfhydryl groups were introduced to the hD1 antibody with N-succinimidyl-S-acetylthiopropionate (SATP; Pierce) and were reduced with hydroxylamine hydrochloride (Pierce) using the manufacturer's protocol. Subsequently, hD1 was added to sSMCC-treated KLH in phosphate-buffered saline (PBS, pH 7.4) and allowed to react for 16 hours at 4°C. Unbound sites were alkylated by adding iodoacetamide (Sigma-Aldrich, St Louis, MO) to a final concentration of 25 mM, followed by 30-minute incubation at room temperature. The protein mixture was loaded onto a Superose 6 column (24-mL bed volume; Amersham Pharmacia Biotech, Uppsala, Sweden), and fractions were collected and analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Fractions containing hD1-KLH were pooled and fractions containing free hD1 were discarded. The efficiency of the cross-linking reaction was estimated by comparing the amount of hD1 relative to KLH before the reaction to the hD1–to–hD1-KLH ratio after cross-linking. We calculated that, on average, each KLH molecule had reacted with 10 hD1 molecules (data not shown). Endotoxin levels of the pooled hD1-KLH fractions were below detection levels (< 0.04 pg/μg protein) in the QCL-1000 *Limulus* amebocyte lysate assay (BioWhittaker, Walkersville, MD).

Monocyte-derived DCs and PBLs

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coats of healthy individuals and were purified using Ficoll density centrifugation. Peripheral blood lymphocytes (PBLs) and immature DCs (iDCs) were obtained from PBMCs as reported elsewhere.[14] In brief, PBMCs were allowed to adhere for one hour at 37°C. Nonadherent cells (PBLs) were gently removed, washed, and cryopreserved. The adherent monocytes were cultured in the presence of interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF, 500 and 800 U/mL, respectively; Schering-Plough International, Kenilworth, NJ) for 6 days to obtain immature DCs. Mature DCs (mDCs) were obtained by culturing iDCs in the presence of 2 μg/mL lipopolysaccharide (LPS) for 24 hours. DCs were cryopreserved until use. Unless indicated otherwise, cells were cultured in X-VIVO 15 medium (Cambrex, Verviers, Belgium) supplemented with 2% human serum.

Tritiated thymidine incorporation assays

Tritiated thymidine (1 μCi [0.037 MBq]/well; MP Biomedicals, Amsterdam, the Netherlands) was added to the cell cultures. Tritiated thymidine incorporation was measured after 16 hours in a β-scintillation counter. Proliferation indices higher than 2 were considered positive.

Clinical vaccination protocol

PBLs and monocyte-derived DCs were isolated from melanoma patients participating in a clinical vaccination trial, as described by de Vries et al.[20] Patients were determined to have stage IV disease according to the American Joint Committee on Cancer (AJCC) criteria.[21] The study was approved by the institutional review board (Radboud University Nijmegen Medical Centre, Commissie Mensgebonden Onderzoek). Informed consent was provided according to the Declaration of Helsinki. The vaccination protocol consisted of 2 parts. In the first part, antigen-pulsed mDCs were administered intravenously and intradermally, 3 times at biweekly intervals. In the second part, patients received 3 monthly intradermal vaccinations with peptides alone (100 μg) and KLH (2 μg). Patients who remained free of disease progression after the first vaccination cycle were eligible for maintenance cycles at 6-month intervals, each consisting of 3 biweekly intranodal vaccinations in a clinically tumor-free lymph node region under ultrasound guidance with mDCs alternately pulsed with wild-type or modified glycoprotein 100 (gp100) peptides,[20] tyrosinase peptides, and KLH. Clinical grade DCs for vaccination purposes were generated from PBMCs as described previously.[22]

Humoral responses to KLH

Humoral responses to KLH were determined by enzyme-linked immunosorbent assays (ELISA) as described by Holtl et al.[23] Briefly, 96-well plates were coated overnight at 4°C with the protein KLH (25 μg/mL) in PBS (0.1

Samsung Bioepis Exhibit No. 1002
Page 7883

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mL/well). Subsequently, plates were incubated with serial dilutions of patient serum, obtained before and during the third maintenance cycle, for one hour at room temperature. After extensive washing, human IgG-specific antibody labeled with horseradish peroxidase was allowed to bind for one hour at room temperature. Peroxidase activity was revealed using 3,3′ 5,5′ tetramethyl-benzidine as substrate and measured in a microtiter plate reader at 450 nm. A signal detected at 1:400 or higher dilution of serum was considered positive.

Cellular responses to KLH

Cryopreserved PBMCs, isolated from peripheral blood samples taken from the patients, were thawed, washed, and plated at 1×10^5 PBMCs per well of a 96-well tissue culture microplate either in the presence or absence of 10 μg/mL KLH. After 4 days of culture, a tritiated thymidine incorporation assay was performed.

Binding and internalization assays

Binding of hD1 and hD1-KLH to iDCs was assessed by immunofluorescence and flow cytometry. iDCs were incubated with or without 10 μg/mL hD1 or hD1-KLH. In some experiments, DC-SIGN was blocked by pretreating iDCs with 100 μg/mL AZN-D1. After a one-hour incubation at 4°C, cells were washed and incubated with Alexa Fluor 647–labeled anti–human IgG antibody. Cells were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences, San Jose, CA).

Internalization of hD1 by iDCs was determined by flow cytometry as described previously.[24] Briefly, iDCs were incubated with 10 μg/mL hD1, AZN-D1, AZN-L19, or mouse IgG1 and 5G1.1 isotype control antibodies at 4°C for 30 minutes, washed, and incubated for 0, 15, 30, or 45 minutes at 37°C. Subsequently, some of the cells were fixed, while others were fixed and permeabilized in PBS/0.1% (vol/wt) saponin (Sigma-Aldrich) before addition of the Alexa Fluor 647–labeled anti–human IgG secondary antibody. The amount of internalized antibody was calculated by subtracting the mean fluorescence in fixed cells (surface bound) from that recorded with fixed and permeabilized cells (internalized and surface bound) at the various time points.

Internalization of hD1 and hD1-KLH was confirmed by confocal laser scanning microscopy (CLSM). iDCs were incubated with 10 μg/mL hD1, hD1-KLH, AZN-D1, or isotype control 5G1.1 and mouse IgG1 antibodies for one hour at 37°C. Cells were fixed on poly-L-lysine–coated glass slides, followed by intracellular staining with Alexa Fluor 647–labeled secondary antibodies. Cells were imaged with a Bio-Rad MRC 1024 confocal system operating on a Nikon Optiphot microscope and a Nikon × 60 planApo 1.4 oil immersion lens (Bio-Rad, Hercules, CA). Pictures were analyzed with Bio-Rad Lasersharp 2000 and Adobe Photoshop 7.0 (Adobe Systems, Mountain View, CA) software.

KLH binding and internalization by iDCs was assessed by direct labeling of KLH using the Alexa Fluor 488 labeling kit (Molecular Probes). iDCs were incubated with 10 μg/mL labeled KLH for one hour at either 4°C or 37°C. Subsequently, cells were washed and analyzed by flow cytometry.

Live imaging of hD1-KLH and KLH uptake by iDCs

iDCs were labeled with LysoTracker Red (Molecular Probes) in PBS for 10 minutes at room temperature. Subsequently, cells were transferred to RPMI 1640 without phenol red (Gibco, Life Technologies, Breda, the Netherlands) supplemented with 1% human serum. Labeled cells were analyzed at 37°C with a Zeiss LSM 510 microscope equipped with a type S heated stage CO_2 controller and PlanApochromatic 63 × 1.4 oil immersion DIC lens (Carl Zeiss, Jena, Germany). KLH and hD1-KLH, directly labeled with the Alexa Fluor 488 labeling kit (Molecular Probes), were added to the medium at 10 μg/mL. Cells were imaged using Zeiss LSM Image Browser version 3.2 (Carl Zeiss) and processed with Image J version 1.32j software (National Institutes of Health, http://rsb.info.nih.gov/ij).

Targeting experiments

iDCs were incubated with hD1, hD1-KLH, or KLH for one hour at 4°C or 37°C. Where indicated, iDCs were matured with LPS. Subsequently, DCs were washed and cocultured with KLH-responsive PBLs (ratio 1:10) at 37°C. In some experiments, iDCs were matured with LPS before addition of PBLs. After 16 hours of coculture, IL-2, IL-4, IL-5, IL-10, tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) were measured by cytometric bead array (CBA) (T helper 1 [Th1]/Th2 Cytokine CBA 1; BD Pharmingen, San Diego, CA) according to the manufacturer's instructions. After 4 days of coculture, a tritiated thymidine incorporation assay was performed.

Presentation of KLH epitopes over time

iDCs were incubated with 5 μg/mL KLH or 5 μg/mL hD1-KLH for one hour at 37°C. Following washing, KLH-responsive PBLs were added to the iDCs either on the same day, or 2 or 4 days later. Four days after addition of the PBLs, proliferative responses were determined in a tritiated thymidine incorporation assay.

Activation and expansion of naive T cells

Experiments were performed essentially as described previously.[7] Briefly, PBLs from a healthy donor were used as a source of T cells. Autologous DCs were incubated with 10 μg/mL hD1-KLH for one hour at 37°C, washed, and matured. Targeted mDCs were incubated with PBLs (ratio, 1:10) in the presence of IL-7 (10 ng/mL, day 0), followed by addition of IL-10 (10 ng/mL) on day 1 and IL-2 (20 U/mL) on day 2. IL-2 was added to the culture every 3 to 4 days. PBLs were restimulated each week. Restimulations were performed with iDCs treated with 5 μg/mL hD1-KLH after 1 week, and with iDCs treated with 2.5 μg/mL hD1-KLH after 2, 3, and 4 weeks. Finally, PBLs were harvested and cocultured with autologous mDCs that had been pulsed with KLH, as described in "Targeting experiments." Proliferative responses were determined by tritiated thymidine incorporation assays.

Results

Humanized anti–DC-SIGN antibody hD1 is internalized by iDCs

The CDRs of the mouse antihuman DC-SIGN antibody AZN-D1 were grafted onto a human IgG2/IgG4 composite antibody to generate a humanized antibody for DC-SIGN targeting, hD1. We have previously shown that the human hybrid IgG2/IgG4 constant domain prevents antibodies from binding to Fc receptors.[17] The binding affinity of hD1 for DC-SIGN was similar to that of AZN-D1 as determined by surface plasmon resonance (3.7 ± 0.7 nM and 3.8 ± 1.1 nM, respectively, data not shown).

Flow cytometric analysis revealed specific binding of hD1 to DC-SIGN on iDCs, as preincubation of iDCs with AZN-D1 efficiently reduced binding (Figure 1A). Furthermore, hD1 bound to DC-SIGN–expressing K562 cells after transfection with DC-SIGN cDNA, whereas it did not bind to untransfected K562 cells (data not shown). A time-course internalization experiment revealed that both the hD1 and the AZN-D1 antibodies were rapidly internalized by iDCs, although the AZN-D1 antibody was internalized slightly more efficiently than hD1. As expected, the control AZN-L19 antibody, directed against CD18, was not internalized (Figure 1B). Analysis by confocal microscopy confirmed internalization of the hD1 and AZN-D1 antibodies by iDCs, whereas the control antibodies 5G1.1, directed against human terminal complement protein C5, and mouse IgG1 isotype were not internalized (Figure 1C).

hD1-KLH is rapidly internalized and translocated to the lysosmal compartment

To further explore the potential of DC-SIGN as a targeting receptor for DC vaccination, we studied the uptake of KLH complexed to

Samsung Bioepis Exhibit No. 1002
Page 7884

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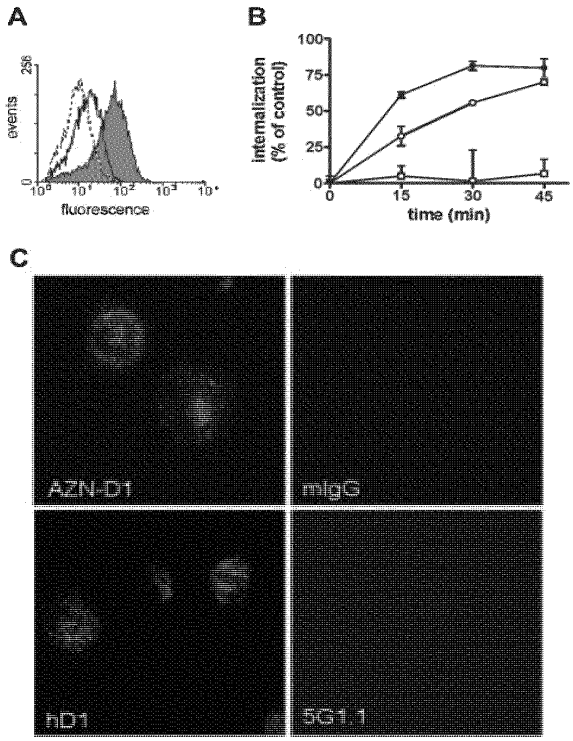


Figure 1. Binding of hD1 to DC-SIGN and internalization by DCs. (A) iDCs were treated with 10 μg/mL hD1 (gray shaded curve), 100 μg/mL AZN-D1 (open dotted curve), or pretreated with 100 μg/mL AZN-D1 followed by 10 μg/mL hD1 incubation (open solid curve), followed by incubation with an Alexa Fluor 647–labeled goat anti–human IgG antibody. Binding of hD1 was analyzed by flow cytometry. (B) iDCs were incubated with AZN-D1 (●), hD1 (○), or AZN-L19 (□) at 4°C for one hour, and transferred to 37°C. Cells were fixed at various time points, and stained with Alexa Fluor–labeled secondary antibodies with or without prior permeabilization. The mean fluorescence was determined by flow cytometric analysis, and the amount of internalized antibody was plotted as a percentage of the amount of total cell-associated antibody. Data represent experiments performed in triplicate ± SD. (C) Internalization of hD1 was confirmed by CSLM. iDCs were incubated with hD1, AZN-D1, or their isotype controls 5G1.1 and mouse IgG1 (mIgG) for one hour at 37°C. Cells were stained with Alexa Fluor 647–labeled secondary antibodies (blue), followed by microscopic analysis. The image represents the middle focal plane of the DCs, with iris set at 2 nm. Original magnification, ×600.

the humanized anti–DC-SIGN antibody hD1. As was observed with the hD1 antibody, hD1-KLH bound to iDCs (Figure 2A). hD1-KLH binding to DCs is mediated by DC-SIGN, as binding could be blocked by pretreating iDCs with AZN-D1 (Figure 2A). Analysis by CLSM revealed that hD1-KLH was rapidly internalized by iDCs (Figure 2B). After one hour of incubation at 37°C, hD1-KLH could be detected in 74% of the iDCs (data not shown). In contrast to hD1-KLH, KLH itself did not bind to iDCs, but was internalized by iDCs following one hour of incubation at 37°C (Figure 2C). These findings demonstrate that internalization of KLH by DCs is not receptor mediated, and likely depends on macropinocytosis.

Foreign antigens are taken up by DCs and shuttled, via endosomes, to the lysosomal compartment where they can be processed. In order to establish whether hD1-KLH reaches the lysosomal compartment, and to study uptake-kinetics and routing of KLH and D1-KLH, these proteins were directly labeled with Alexa Fluor 488 dye. Live imaging by CLSM revealed rapid uptake of hD1-KLH and KLH by iDCs. Both hD1-KLH and KLH were detected in the lysosomal compartment within one hour after their addition to the culture medium (Figure 2D; Supplemental Videos S1 and S2; see the Supplemental Videos link at the top of the online article, at the *Blood* website).

Induction of KLH responses by DC vaccination

In order to compare the antigen-presentation capacity of DCs pulsed with KLH to that of DCs targeted by hD1-KLH, we required KLH-specific T cells. To this end, PBLs were isolated from melanoma patients participating in a vaccination study. This study involved vaccination of patients with autologous DCs pulsed ex vivo with KLH and melanoma-associated antigens. Monocyte-derived DCs and PBLs from patients showing humoral as well as cellular responses against KLH were used for the targeting studies.

All 31 vaccinated patients participating in the clinical vaccination trial showed responses against KLH (data not shown). Humoral responses were readily detected in the patients' serum. KLH-specific IgGs were present after the first vaccination cycle, and their serum levels increased upon the second vaccination (Figure 3A). Cellular responses were analyzed using PBMCs obtained from patients one week before the start of the third vaccination cycle. Typically, PBMCs pulsed with KLH showed an increased proliferative response compared with unpulsed PBMCs, revealing the presence of KLH-reactive T cells in peripheral blood

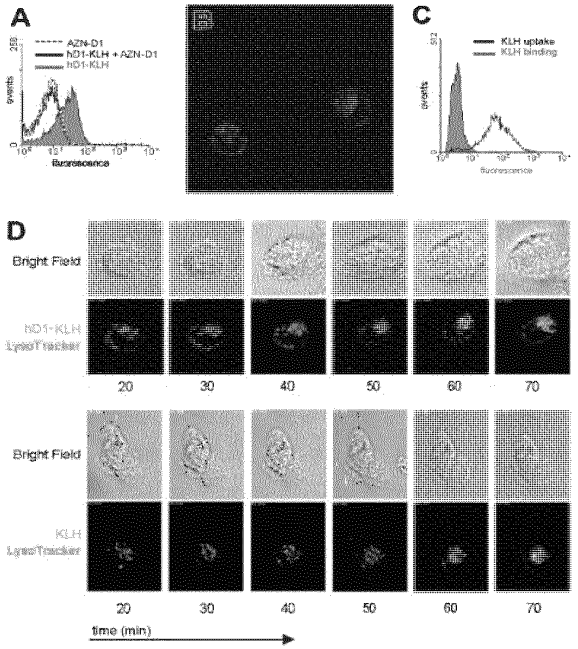


Figure 2. Binding and uptake of hD1-KLH by DCs. (A) iDCs were treated with 10 μg/mL hD1-KLH, 100 μg/mL AZN-D1, or pretreated with 100 μg/mL AZN-D1 followed by 10 μg/mL hD1 incubation, followed by incubation with an Alexa Fluor 647–labeled goat anti–human IgG antibody. Binding of hD1-KLH was analyzed by flow cytometry. (B) Internalization of hD1-KLH was confirmed by CSLM. iDCs were incubated with Alexa Fluor 647–labeled hD1-KLH (blue) for one hour at 37°C, followed by microscopic analysis. The image represents the middle focal plane of the DCs, with iris set at 2 nm. Original magnification, ×600. (C) iDCs were incubated with Alexa Fluor 488–labeled KLH for one hour at 4°C or 37°C. Subsequently, cells were fixed and analyzed by flow cytometric analysis. (D) iDCs were labeled with LysoTracker Red, followed by addition of Alexa Fluor 488–labeled (green) hD1-KLH or KLH. Cells were imaged by CSLM. Data represent bright field and corresponding fluorescent images of cells at various time points after addition of hD1-KLH or KLH to the culture medium. Original magnification, ×630.

Samsung Bioepis Exhibit No. 1002
Page 7885

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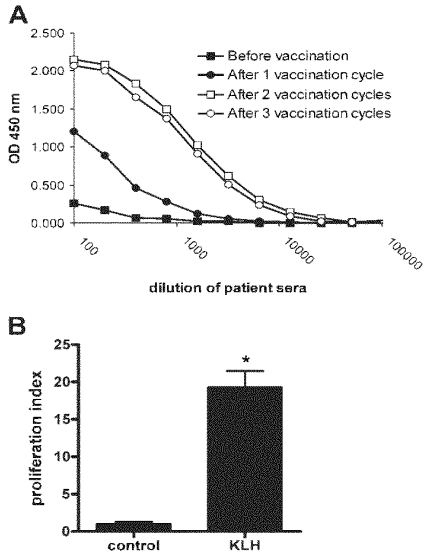


Figure 3. Humoral and cellular responses against KLH in patients vaccinated with KLH-pulsed DCs. (A) Patient serum was obtained both before the first, and after the first, second, and third vaccination cycles. Total IgG antibodies specific for KLH were detected by ELISA. Data represent optical density (OD) 450 values of serially diluted serum samples for a representative patient. (B) Patient PBMCs were isolated one week prior to the third vaccination cycle and cultured in the absence (control) or presence of 10 μg/mL KLH (KLH). Cellular responses were assessed in a tritiated thymidine incorporation assay. Data represent mean ± SD of experiments performed in 6-fold for a representative patient. Significant difference from control according to Student *t* test: $^*P < .001$.

(Figure 3B). These findings show that KLH-pulsed mDCs effectively induced both humoral and cellular responses in vivo.

Targeting of KLH to DC-SIGN results in presentation of KLH antigen epitopes

To demonstrate that antibody-mediated targeting of antigen to DC-SIGN leads to antigen presentation, autologous iDCs were incubated with 5 μg/mL KLH (± 0.63 nM) or hD1-KLH (± 0.53 nM) at 4°C for one hour, washed to remove unbound protein, and added to PBLs. Presentation of KLH epitopes to specific T cells was determined by measuring proliferative responses (Figure 4A-B) and the levels of cytokines secreted in the medium (Figure 4C). As endocytotic processes are inhibited at 4°C, and KLH does not bind to iDCs (Figure 2C), hD1-KLH can be internalized only after binding to DC-SIGN. iDCs incubated with hD1-KLH induced cellular responses by the PBLs, whereas iDCs incubated with KLH did not (Figure 4A-C), showing that targeting of KLH to DC-SIGN results in antigen presentation. We have previously demonstrated that the clinical vaccination protocol used in our studies results in a Th1-type immune response.[20] Indeed, stimulation of patient PBLs with hD1-KLH–treated iDCs resulted in enhanced secretion of IL-2, IFN-γ, and TNF-α (Figure 4C), while IL-4, IL-5, and IL-10 levels were not detectable (data not shown). As expected, iDCs incubated with KLH at 37°C induced proliferative responses, as did iDCs incubated with hD1-KLH (Figure 4A).

Presentation of KLH epitopes by iDCs incubated with hD1-KLH at 4°C was abolished by preincubation of iDCs with the hD1 antibody, but was unaffected by preincubation with the isotype control 5G1.1 (Figure 4B-C). This confirms that hD1-KLH targeted specifically to DC-SIGN, resulting in presentation of KLH epitopes. Cross-linking of hD1 to KLH was required for induction of DC-SIGN–mediated presentation of KLH epitopes, since iDCs incubated with both KLH and hD1 antibody did not induce cellular responses (Figure 4B-C).

Targeting KLH to DC-SIGN enhances its immunogenicity

To determine whether DC-SIGN targeting increases the efficiency of antigen presentation, immature monocyte-derived DCs from previously immunized patients were incubated at 37°C with various concentrations of KLH or hD1-KLH. Subsequently, iDCs were washed, and a portion of the iDCs was matured with LPS. hD1-KLH by itself did not induce maturation of iDCs (data not shown). Both hD1-KLH–treated iDCs (Figure 5A) and mDCs (Figure 5B) induced cellular responses at 100-fold lower concentrations than KLH-pulsed DCs. Thus, antigen targeting via DC-SIGN significantly enhances immunogenicity.

KLH epitopes are presented up to 4 days after targeting DC-SIGN

After antigen uptake in the periphery, DCs migrate to the draining lymph nodes to present the processed antigen to T cells. It is

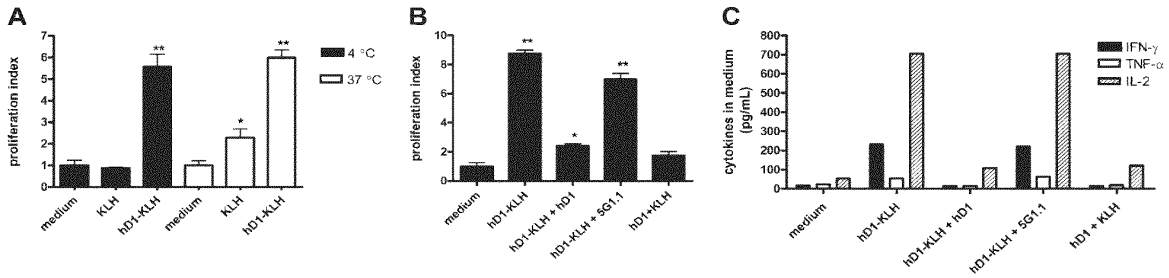


Figure 4. Antibody-mediated targeting of antigen to DC-SIGN results in antigen presentation. (A) iDCs were incubated with medium (control), 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 4°C or 37°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from the patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to analysis of variance (ANOVA) and Bonferroni test: $^*P < .05$; $^{**}P < .001$. (B) iDCs were incubated with medium (control), 5 μg/mL hD1-KLH, 5 μg/mL hD1-KLH and 100 μg/mL hD1 (hD1-KLH + hD1), 5 μg/mL hD1-KLH and 100 μg/mL 5G1.1 (hD1-KLH + 5G1.1), or 5 μg/mL hD1 and 5 μg/mL KLH (hD1 + KLH) for one hour at 4°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from medium control according to ANOVA and Dunnett test: $^*P < .05$; $^{**}P < .01$. (C) Production of IFN-γ, TNF-α, and IL-2 in the coculture experiment described in panel B. After 16 hours of coculturing iDCs and PBLs, supernatants were taken and cytokine levels were determined. Data represent cytokine levels in pooled samples of experiments performed in triplicate.

Samsung Bioepis Exhibit No. 1002
Page 7886

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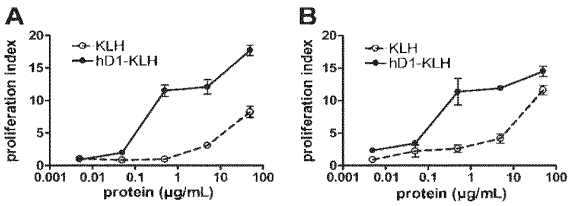


Figure 5. Targeting of antigen to DC-SIGN enhances its immunogenicity. (A) iDCs were incubated with medium, 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 37°C. Subsequently, iDCs were washed and cocultured with autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. After 4 days, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. (B) Experiment performed as described in panel A, except that antigen-treated iDCs were matured with LPS before addition of PBLs.

imperative that DCs present the antigens over a prolonged period of time to initiate a significant immune response. We evaluated the efficiency of antigen presentation over time following targeting by incubating patient PBLs with autologous iDCs that had been pulsed with KLH or hD1-KLH 0, 2, or 4 days earlier. The results demonstrate that DCs targeted with hD1-KLH presented KLH epitopes up to 4 days after targeting. Moreover, at all time points evaluated, DCs targeted with hD1-KLH induced stronger proliferative responses than DCs pulsed with KLH (Figure 6).

hD1-KLH targeted to DCs activates both major histocompatibility complex (MHC) class I– and II–restricted naive T cells recognizing KLH epitopes

To evaluate whether hD1-KLH, besides triggering recall responses, is able to activate naive T cells, hD1-KLH was tested in an autologous in vitro culture system. PBLs from a healthy donor were stimulated by repetitive coculturing with hD1-KLH–pulsed autologous DCs. IL-10 was added to the cultures to stimulate expansion of $CD8^+$ T cells.[25] After 5 rounds of stimulation, PBLs were cocultured with DCs pulsed with KLH or hD1 to evaluate KLH-specific proliferative responses. The results demonstrate that hD1-KLH–targeted DCs activated KLH-specific naive T cells (Figure 7). Presentation of KLH epitopes was mediated by MHC class I and class II, since both class I and class II blocking antibodies significantly reduced the proliferative response, while an antibody control did not. DCs pulsed with hD1 antibody did not induce proliferative responses by the PBLs (Figure 7), demonstrating that the T-cell response is specific for KLH epitopes rather than epitopes contained within the hD1 antibody.

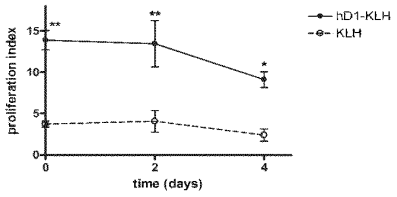


Figure 6. Antigen targeted to DC-SIGN is presented for at least 4 days. iDCs were incubated with medium, 5 μg/mL KLH, or 5 μg/mL hD1-KLH for one hour at 37°C. Subsequently, iDCs were washed and cultured for 0, 2, or 4 days before addition of autologous KLH-responsive PBLs, derived from patients enrolled in the vaccination trial. Four days after addition of PBLs, cellular responses were assessed in a proliferation assay. Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference from KLH according to ANOVA and Bonferroni test: $^*P < .05$; $^{**}P < .01$.

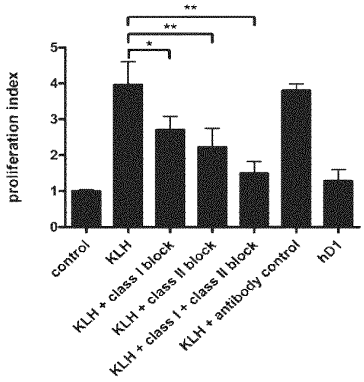


Figure 7. hD1-KLH–treated DCs activate naive T cells recognizing KLH epitopes. iDCs derived from a healthy donor were incubated with medium (control), 10 μg/mL KLH, or 10 μg/mL hD1 for one hour at 37°C. Subsequently, cells were washed and matured with LPS. mDCs were cocultured with autologous PBLs that had been repeatedly stimulated with DCs treated with hD1-KLH as described in "Patients, materials, and methods." Some cocultures of KLH-pulsed mDCs and PBLs were supplemented with w6/32 antibody (KLH + class I block), IVA-12 (KLH + class II block), both w6/32 and IVA-12 (KLH + class I + class II block), or total mouse IgG (KLH + antibody control). Data are mean proliferation indices relative to medium control for experiments performed in triplicate ± SD. Significant difference according to ANOVA, followed by the Student-Newman-Keuls test: $^*P < .01$; $^{**}P < .001$.

Discussion

In the present study, we explored the capacity of the type II C-type lectin DC-SIGN to function as a target receptor for vaccination purposes. The results demonstrate that antibody-mediated targeting of antigen to human DC-SIGN was 100-fold more efficient than pulsing DCs with antigen, and that antigen epitopes were presented for at least 4 days after targeting.

A critical step in designing a suitable targeting construct is the choice of targeting antibody. Various antibodies directed against the same receptor might bind distinct receptor epitopes, thus influencing biologic outcome. For example, not all antibodies directed against DC-SIGN are internalized following binding,[13] and the anti-MR antibody PAM-1 induces maturation of iDCs, in contrast to a different isotype-matched anti-MR antibody.[26] The mouse antihuman DC-SIGN antibody AZN-D1 has been previously described to be internalized by DCs,[13] a prerequisite for successful targeting. However, the use of murine antibodies in humans presents numerous problems, including a short half-life and high immunogenicity.[27] We were able to graft the CDR of AZN-D1 hypervariable domains onto a human composite IgG2/IgG4 antibody without loss of binding characteristics. The resulting hD1 antibody exhibited a similar binding affinity for DC-SIGN as AZN-D1, and did not induce maturation of iDCs (data not shown). hD1 binding induced rapid internalization of DC-SIGN, while internalization did not result in a lasting down-regulation of DC-SIGN expression (data not shown), thus minimizing the effect a therapeutic intervention might have on the biologic function of DC-SIGN. hD1 rapidly targeted antigen to the DC lysosomal compartment resulting in antigen presentation, and did not induce hD1-specific proliferative responses in the naive T-cell stimulation experiments. Thus, humanization of AZN-D1 resulted in an effective targeting antibody, and will facilitate introduction into clinical trials.

Vaccination strategies aimed at inducing cytotoxic T-cell help, such as antitumor therapies, require antigen presentation in the

Samsung Bioepis Exhibit No. 1002
Page 7887

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context of MHC class I. In addition, the induction of CTL responses requires bystander $CD4^+$ T-cell help.[28] Targeting DCs with hD1-KLH resulted in the activation of naive T cells in the context of both MHC class I and II. Although the major route for presentation of exogenous antigens is via class II, presentation via class I can occur via the process of cross-presentation. This process provides internalized proteins access to cytosolic proteasomes and their derived peptides access to the endoplasmatic reticulum (ER)–based class I processing machinery.[29] Particulate antigens taken up by phagocytosis have access to this machinery since phagosomes fuse with the ER soon after or during their formation.[30] Particulate antigens are more efficiently cross-presented than soluble antigens.[31,32] However, a recent study by Ackerman et al reveals that internalized soluble proteins can escape proteolysis and also gain access to the lumen of the ER.[33] This might explain how antibody-mediated targeting of the type I C-type lectins MR[7] and DEC-205[10] results in class I responses. Although we cannot exclude the possibility that, besides receptor-mediated endocytosis, a portion of our chimeric protein was taken up via macropinocytosis, our current findings strongly suggest that antibody-mediated targeting of DC-SIGN results in cross-presentation.

The technique commonly used in current clinical trials to load DCs with antigens involves ex vivo incubation with MHC class I and II binding peptides. Other techniques involve loading with tumor lysates or apoptotic tumor cells, and the introduction of genetic material to drive expression of specific antigens by the DC itself.[2] Ideally, vaccines should deliver antigens to the DCs in vivo. Antibody-mediated targeting of antigens to DC surface receptors to stimulate antigen presentation in vivo has been shown to be far more potent than immunization with antigen in complete Freund adjuvant or splenic DCs pulsed with antigen ex vivo.[6] However, mere targeting of antigens to DCs is not sufficient for induction of immunity. Antibody-mediated targeting of antigen to DEC-205 in mice leads to tolerance, and coadministration of a DC maturation stimulus is required to induce immunity.[6,10,11] These findings are consistent with DC-based vaccination studies in humans, showing that DC maturation is a prerequisite for induction of immunity.[20] Vaccination strategies for transplantation, allergy, autoimmunity, and chronic inflammatory diseases could exploit the finding that targeted iDCs induce tolerance. However, strategies aimed at inducing immunity will require a combination of antigens and DC activation factors. Agents that have been shown to activate DCs in vivo include anti-CD40 antibody,[11] α-galactosylceramide,[34] and the Toll-like receptor ligands LPS[35] and cytosine-phosphate-guanosine (CpG) oligonucleotides.[36] A better understanding of differences in DC subsets, their activation pathways, and antigen uptake receptors will provide the information necessary for development of effective vaccines.

In the present study, we used a neoantigen to study targeting efficiency. A major challenge in cancer therapy is to break immunologic tolerance to tumor-associated self-antigens. Tolerance can be the result of clonal deletion, active suppression of antigen-specific T cells by regulatory T cells, or inadequate activation stimuli provided by tumor cells upon antigen presentation, resulting in T-cell anergy.[37] One way of breaking tolerance is by disruption of negative regulatory mechanisms directly at the T-cell level. Antibody-mediated blockade of the cytotoxic T lymphocyte–associated antigen 4 (CTLA-4) breaks tolerance against moderately immunogenic tumors[38] and improves the efficiency of tumor cell–based vaccines against poorly immunogenic tumors.[39] Depletion of $CD25^+$ regulatory T cells prior to blocking of CTLA-4 improves vaccine efficiency even further.[40] A second way of breaking tolerance is to recruit DCs as potent APCs. DCs can overcome the heightened threshold of anergic T cells[41] and restore responsiveness of tolerogenic tumor-specific T cells[42] in vitro. Moreover, studies in mice demonstrate that DC-based vaccines can break tolerance against (tumor-associated) self-antigens,[43-47] resulting in regression of established tumors.[47] Thus, a combination of antibody-mediated strategies targeting tumor-associated antigens to DCs and strategies modulating regulatory mechanisms at the T-cell level might provide effective cancer therapies.

In conclusion, antibody-mediated targeting of antigens to DC surface receptors represents an exciting way to induce immune responses. Our results demonstrate efficient delivery of antigen to DCs via DC-SIGN, resulting in naive as well as recall responses by T cells. These data expose DC-SIGN as a promising target molecule for antibody-mediated antigen delivery to DCs.

Acknowledgments

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Samsung Bioepis Exhibit No. 1002
Page 7888

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Samsung Bioepis Exhibit No. 1002
Page 7889

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Effective induction of naive and recall T-cell responses by targeting antigen to human dendritic cells via a humanized anti–DC-SIGN antibody

Paul J. Tacken, I. Jolanda M. de Vries, Karlijn Gijzen, Ben Joosten, Dayang Wu, Russell P. Rother, Susan J. Faas, Cornelis J. A. Punt, Ruurd Torensma, Gosse J. Adema and Carl G. Figdor

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Samsung Bioepis Exhibit No. 1002
Page 7890

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The Efficacy and Safety of Rituximab in Patients With Active Rheumatoid Arthritis Despite Methotrexate Treatment

Results of a Phase IIb Randomized, Double-Blind, Placebo-Controlled, Dose-Ranging Trial

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Objective. **To examine the efficacy and safety of different rituximab doses plus methotrexate (MTX), with or without glucocorticoids, in patients with active rheumatoid arthritis (RA) resistant to disease-modifying antirheumatic drugs (DMARDs), including biologic agents.**

Methods. **A total of 465 patients were randomized into 9 treatment groups: 3 rituximab groups (placebo [n = 149], 500 mg [n = 124], or 1,000 mg [n = 192] on days 1 and 15) each also taking either placebo glucocorticoids, intravenous methylprednisolone premedication, or intravenous methylprednisolone premedication plus oral prednisone for 2 weeks. All patients received MTX (10–25 mg/week); no other DMARDs were permitted.**

Results. **Significantly more patients who received 2 500-mg or 2 1,000-mg infusions of rituximab met the American College of Rheumatology 20% improvement criteria (achieved an ACR20 response) at week 24 (55% and 54%, respectively) compared with placebo (28%; $P < 0.0001$). ACR50 responses were achieved by 33%, 34%, and 13% of patients, respectively ($P < 0.001$), and ACR70 responses were achieved by 13%, 20%, and 5% of patients ($P < 0.05$). Changes in the Disease Activity Score in 28 joints (−1.79, −2.05, −0.67; $P < 0.0001$) and moderate to good responses on the European League Against Rheumatism criteria ($P < 0.0001$) reflected the ACR criteria responses. Glucocorticoids did not contribute significantly to the primary efficacy end point, ACR20 response at 24 weeks. Intravenous glucocorticoid premedication reduced the frequency and intensity of first infusion–associated events; oral glucocorticoids conferred no additional safety benefit. Rituximab was well tolerated; the type and severity of infections was similar to those for placebo.**

Conclusion. **Both rituximab doses were effective and well tolerated when added to MTX therapy in patients with active RA. The primary end point (ACR20 response) was independent of glucocorticoids, although intravenous glucocorticoid premedication improved tolerability during the first rituximab infusion.**

Although cytokine-specific biologic agents have improved the treatment of rheumatoid arthritis (RA), some patients have an inadequate response to such therapies and, in clinical practice, most have only a

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Samsung Bioepis Exhibit No. 1002
Page 7891

partial response (1). The frequency of remission has remained <50% for patients with established RA, many of whom display "moderate" disease activity, according to their Disease Activity Score (DAS) (2). In order to achieve additional significant gains in RA therapy, new targets need to be identified. One such target is the B cell (3).

Rituximab is a monoclonal antibody that selectively depletes CD20+ B cells via 3 putative mechanisms: antibody-dependent cell-mediated cytotoxicity, complement-dependent cytotoxicity, and promotion of CD20+ B cell apoptosis (4–6). CD20 is not expressed on stem cells or plasma cells (7,8); this is consistent with the observation that B cell recovery and Ig production were not compromised after a single course of rituximab (9).

Rituximab has shown efficacy in the treatment of CD20+ B cell non-Hodgkin's lymphoma (NHL) (10). More recently, a role for rituximab and B cell modulation has been investigated in rheumatoid factor (RF)–positive patients with active RA. In a phase IIa study, 2 1,000-mg infusions of rituximab (combined with methotrexate [MTX] and a 2-week treatment with glucocorticoids) significantly improved RA symptoms for up to 1 year compared with MTX alone (9). Moderate to good responses according to the European League Against Rheumatism (EULAR) criteria were also evident at 2 years in 33% of the rituximab plus MTX group (11).

To date, no studies have investigated the influence of the rituximab dosage or the role of glucocorticoids in the clinical efficacy and/or tolerability of rituximab regimens in RA. The objective of this study was therefore to examine these variables in patients with active RA who had an inadequate response to disease-modifying antirheumatic drugs (DMARDs) other than MTX or biologic agents, and who currently had an inadequate clinical response to MTX.

PATIENTS AND METHODS

Role of the funding source. This study was funded by Roche, and Roche scientists contributed to the study design, collection of data, and data analysis. The manuscript was written by Dr. Emery. The agreement to submit the manuscript and the approval of the content were made jointly by the authors.

Study design. This phase IIb, randomized, double-blind, double-dummy, placebo-controlled, international multifactorial trial of 9 different treatment regimens examined 2 dosage levels (and rituximab placebo) of both rituximab and glucocorticoids in a 3 × 3 configuration (Figure 1) with concomitant MTX in patients with active RA. Patients were evaluated at 4-week intervals between baseline and week 24.

Patients. Outpatients were eligible for study if they were between 18 and 80 years of age and had presented at least 6 months prior to randomization with moderate or severe RA

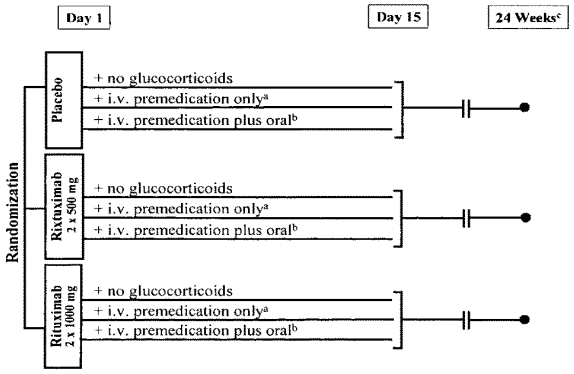


Figure 1. Study design. All groups received weekly methotrexate 10–25 mg parenterally or orally. a = 100 mg methylprednisolone administered intravenously (IV) prior to rituximab infusions on days 1 and 15. b = 100 mg methylprednisolone administered IV prior to rituximab infusions on days 1 and 15, and prednisone administered orally at 60 mg on days 2–7 and at 30 mg on days 8–14. c = primary end point: percentage of patients who met the American College of Rheumatology 20% improvement criteria at week 24.

(diagnosed according to the American College of Rheumatology [ACR; formerly, the American Rheumatism Association] revised criteria [22]) despite ongoing treatment with MTX at a dosage of 10–25 mg/week (orally or parenterally) for at least 12 weeks before randomization, with a stable dosage during the last 4 weeks. Active disease was defined as a swollen and tender joint count ≥8 and either an erythrocyte sedimentation rate ≥28 mm/hour or a C-reactive protein (CRP) serum level ≥1.5 mg/dl.

Patients must have failed prior treatment, manifesting as a lack or loss of response to treatment with at least 1 but not more than 5 DMARDs (other than MTX) and/or biologic response modifiers. Patients discontinued DMARDs (except MTX) and biologic therapy at least 4 weeks before randomization and discontinued infliximab, adalimumab, or leflunomide at least 8 weeks before randomization. Patients who were already taking oral glucocorticoids (≤10 mg/day of prednisone or equivalent) were included if the dosage had been stable for at least 4 weeks before trial entry, and patients who had received intraarticular or parenteral glucocorticoids were included, provided that the most recent administration was >4 weeks before screening. Concomitant treatment with nonsteroidal antiinflammatory drugs was permitted if the dosage had been stable for at least 2 weeks prior to entry. Concomitant treatment with any DMARD (other than MTX), anti–tumor necrosis factor α, or other biologic therapy was prohibited.

Patients were excluded if they had significant systemic involvement secondary to RA, evidence of significant other illnesses or laboratory abnormalities, a history of severe allergic or anaphylactic reactions to humanized or murine monoclonal antibodies, or previous treatment with rituximab or any lymphocyte-depleting therapies. In addition, patients were excluded if they had a history of recurrent significant infection.

The study was performed in accordance with the Declaration of Helsinki. All participating sites received ap-

Samsung Bioepis Exhibit No. 1002
Page 7892

Table 1. Randomization of trial medication and disposition of patients at 24 weeks*

	Treatment†		
	Rituximab placebo, RF+/RF−	Rituximab 2 500-mg doses, RF+/RF−	Rituximab 2 1,000-mg doses, RF+/RF−
Glucocorticoid placebo	42/21	41/0	43/22
IV glucocorticoid premedication‡	42/0	41/0	42/20
IV glucocorticoid premedication + oral glucocorticoid§	44/0	42/0	43/22
Total no. of patients randomized¶	149	124	192
Patients withdrawn, no. (%)	52 (35)	11 (9)	27 (14)
Insufficient response	46 (31)	8 (6)	16 (8)
Adverse events	0	3 (2)	6 (3)
All other reasons#	6 (4)	0	5 (3)
Completed 24 weeks, no. (%)	97 (65)	113 (91)	165 (86)

* RF = rheumatoid factor; IV = intravenous.
† Rituximab or rituximab placebo was administered as 2 infusions: 1 on day 1 and 1 on day 15. All patients received 10–25 mg of methotrexate orally or parenterally on a weekly basis.
‡ Methylprednisolone 100 mg IV on days 1 and 15.
§ Methylprednisolone 100 mg IV on days 1 and 15 plus oral prednisone 60 mg on days 2–7 and 30 mg on days 8–14 (total oral prednisone dose 570 mg).
¶ Patients received at least 1 dose of rituximab.
In the rituximab placebo and rituximab groups receiving 2 1,000-mg infusions, 4 patients (3%) and 4 patients (2%), respectively, refused treatment.

proval from their governing institutional review board (or equivalent). See Appendix A for additional members of the Dose-Ranging Assessment: International Clinical Evaluation of Rituximab in Rheumatoid Arthritis (DANCER) Study Group and their locations. All patients provided written informed consent. The primary study population for efficacy analysis was composed of RF-positive patients. A limited number of RF-negative patients were also included for an initial exploratory analysis of the safety of rituximab in this group.

Study treatment. Rituximab was administered by intravenous (IV) infusion in RF-positive patients: placebo, 500 mg or 1,000 mg on days 1 and 15 (total dose 0 mg, 1,000 mg, and 2,000 mg). Glucocorticoids were administered as placebo methylprednisolone, given IV 30–60 minutes before the infusion of rituximab (or rituximab placebo) on days 1 and 15, premedication methylprednisolone 100 mg, given IV on days 1 and 15 (250 mg prednisone equivalent), or premedication methylprednisolone 100 mg, given IV on days 1 and 15 plus 60 mg of oral prednisone on days 2–7 and 30 mg on days 8–14 (total glucocorticoid dose 820 mg prednisone equivalent) (Table 1). RF-negative patients received either placebo or rituximab (2 1,000-mg infusions), with or without glucocorticoids. All patients received a weekly regimen of MTX (10–25 mg orally or parenterally) with folate (≥5 mg/week).

Trial end points. The primary end point of the trial was the proportion of RF-positive patients who met the ACR 20% improvement criteria (achieved an ACR20 response) (defined as a 20% improvement in the tender and swollen joint counts and a 20% improvement in 3 of the 5 other ACR improvement criteria) (13) at week 24. Secondary and exploratory analyses examined group differences in ACR50 and ACR70 responses, DAS 28-joint assessment for swelling and tenderness (DAS28) (14), EULAR responses (15), fatigue, as measured by the Functional Assessment of Chronic Illness Therapy–Fatigue (FACIT-F) subscale (16), and the effect on individual parameters of the ACR improvement criteria. The level of physical functioning and disability was assessed using the disability index (DI) of the Health Assessment Questionnaire (HAQ) (17).

Safety outcomes were expressed as the incidence of adverse events, graded according to National Cancer Institute (NCI) Common Toxicity Criteria (CTC), version 2.0 (18). CD19+ counts, measured by fluorescence-activated cell sorting, were used as a marker of the onset and duration of peripheral B cell depletion (7). Effects on Ig levels, protective antibody titers, and human antichimeric antibody (HACA) levels were also evaluated.

Statistical analysis. The trial was planned to recruit 440 patients: 40 RF-positive patients per group (a total of 360 patients), who constituted the primary efficacy population, and 20 RF-negative patients in 4 of the 9 groups (a total of 80 patients), for exploratory analysis. Using a 2-sided 5% significance level, it was calculated that a sample size of 360 RF-positive patients would provide at least 80% power to detect differences between the proportions of patients who achieved an ACR20 response in the rituximab 2 1,000-mg and 2 500-mg infusion groups, as well as dose-response trends across the 3 pooled rituximab groups. Randomization was stratified by region (US versus non-US) and by RF status. Steroids were included in the main effects model of ACR20 response at 24 weeks to determine whether a significant effect of the steroid regimen had been achieved. The study was powered to test for an association between ACR20 response at week 24 and rituximab treatment while controlling for corticosteroid usage and region (US or non-US) for RF-positive patients, using a logistic regression model (main effects model). It was not powered to determine any individual effects of these controlling factors.

Primary analyses were based on intent-to-treat (ITT), with nonresponders imputed for all categorical end points (e.g., ACR and EULAR responses), and the last observation was carried forward for continuous variables (e.g., ACR improvement criteria and DAS28). All patients who dropped out of the study were considered nonresponders for purposes of categorical end points. A main-effect logistic regression model

Samsung Bioepis Exhibit No. 1002
Page 7893

Table 2. Patient demographics and baseline disease characteristics*

	Rituximab placebo	Rituximab 2 500-mg infusions	Rituximab 2 1,000-mg infusions
Safety population, no.	149	124	192
Demographics			
Female, %	80	83	80
Age, mean years	51.1	51.4	51.1
Duration of RA, mean years	9.3	11.1	10.8
Previous DMARDs (other than MTX), mean no.	2.2	2.5	2.5
Prior anti-TNFα treatment, %	26	33	28
MTX dose, mean mg/week	15.6	16.0	14.9
US patients, %	33	33	34
Primary ITT population, no.	122	123	122
Disease characteristics			
Swollen joint count, mean no.	21	22	22
Tender joint count, mean no.	35	33	32
Baseline HAQ DI score, mean	1.7	1.8	1.7
RF, mean IU/liter	437	421	437
ESR, mean mm/hour	40	45	41
Serum CRP, mean mg/dl	3.3	3.2	3.0
DAS28, mean	6.8	6.8	6.7

* RA = rheumatoid arthritis; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; TNFα = tumor necrosis factor α; ITT = intent to treat; HAQ = Health Assessment Questionnaire; DI = Disability Index; RF = rheumatoid factor; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; DAS28 = Disease Activity Score in 28 joints.

was used to evaluate ACR20 response at week 24 in the primary efficacy population across all study groups, with terms for rituximab, glucocorticoid usage, and region (US or non-US). This model also studied any effect of adding RF-negative patients to the analysis population. Contrast tests were conducted for rituximab 2 1,000-mg infusions versus placebo, and rituximab 2 500-mg infusions versus rituximab 2 1,000-mg infusions, in addition to a dose-response relationship in the ACR20 response across the upper- and lower-dose rituximab and placebo groups. No comparisons between the rituximab 2 500-mg infusions and placebo were originally planned.

RESULTS

Patient demographics and characteristics. A total of 465 patients were treated, 380 RF-positive patients and 85 RF-negative patients; this constituted the safety population (Table 1). Patients in each of the rituximab groups were generally well matched in terms of demographic parameters and region, baseline disease characteristics, and previous DMARD/biologic treatments (Table 2). Antimalarial agents had been taken by 38–68% of patients across treatment groups, sulfasalazine by 38–68% and immunosuppressive agents (cyclosporine, azathioprine, and/or leflunomide) by 24–44%. Overall, 29% of patients had previously taken biologic agents (infliximab, adalimumab, etanercept, or anakinra), with no significant differences between the randomized groups.

Of the 465 patients, 65% in the placebo group, 91% in the rituximab 2 500-mg infusions group, and 86% in the rituximab 2 1,000-mg infusions group completed the 24-week primary analysis period (Table 1). Fewer patients discontinued rituximab than placebo as a result of insufficient therapeutic response, but rituximab was associated with more withdrawals because of adverse events. Nine patients (all in rituximab groups) withdrew for safety reasons (mainly as a result of infusion-related events). One patient in the 2 500-mg rituximab infusions plus IV glucocorticoid group died due to a cerebrovascular accident.

The primary ITT efficacy population was composed of 367 RF-positive patients; the remaining RF-positive patients were excluded from this analysis for reasons related to accidental unblinding, drug dispensing errors, and unverifiable data.

Efficacy outcomes. The proportion of patients in the primary ITT efficacy population who achieved an ACR20 response at 24 weeks, the primary end point of the trial, was significantly greater in each of the rituximab groups than in the placebo group ($P < 0.0001$) (Figure 2A). At week 24, 55% of the patients in the rituximab 2 500-mg infusions group and 54% of those in the 2 1,000-mg infusions group had achieved an ACR20 response, compared with 28% in the placebo group. There was no statistically significant difference in the odds of achieving an ACR20 response between the groups taking 2 1,000-mg infusions of rituximab versus 2 500-mg infusions of rituximab (odds ratio 0.93, $P = 0.768$). Analyses of the proportions of patients who achieved an ACR50 or ACR70 response showed a similar pattern. Compared with placebo, a significantly greater proportion of patients treated with either dosage

Samsung Bioepis Exhibit No. 1002
Page 7894

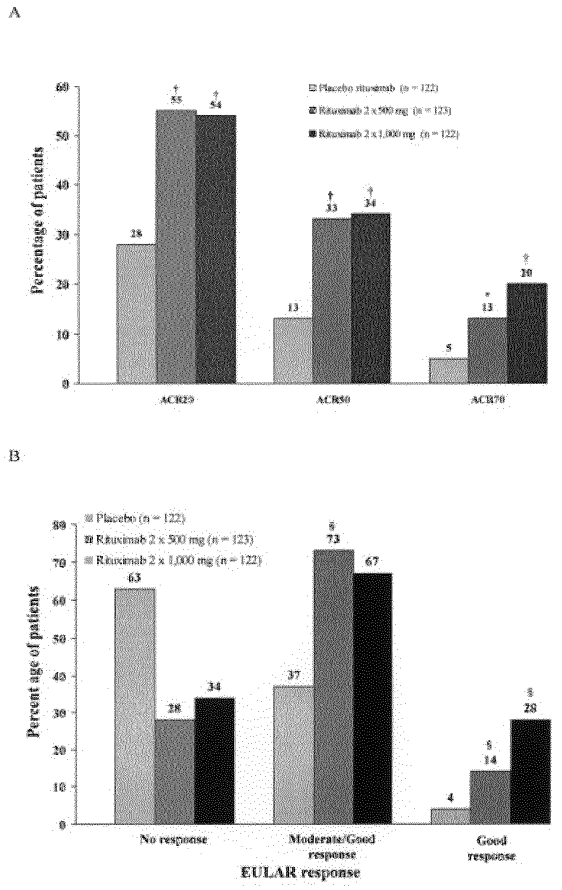


Figure 2. Patients who experienced responses according to **A,** the American College of Rheumatology 20% (ACR20), 50% (ACR50), and 70% (ACR70) improvement criteria and **B,** the European League Against Rheumatism (EULAR) criteria at 24 weeks. Results are expressed using pooled data for glucocorticoid dose. Patients who withdrew or who had insufficient data from which to calculate an ACR score were classified as nonresponders. * = $P = 0.029$; † = $P \leq 0.001$; § = $P < 0.0001$, versus placebo.

of rituximab achieved an ACR50 response ($P \leq 0.001$ versus placebo in each rituximab dosage group) and an ACR70 response ($P = 0.029$ for rituximab 2 500-mg infusions group and $P \leq 0.001$ for rituximab 2 1,000-mg infusions group). Although ACR20 and ACR50 responses were similar for both rituximab dosages, ACR70 responses were numerically more frequent in the rituximab 2 1,000-mg infusions group than in the 2 500-mg infusions group (Figure 2A).

Primary analysis within the main-effect model showed that glucocorticoid treatment had no significant effect on ACR20 response ($P = 0.166$). Dose-contrast tests between individual glucocorticoid treatments showed that the probability of achieving an ACR20 response in any of the main treatment groups was not significantly different for glucocorticoid placebo versus IV glucocorticoids ($P = 0.62$) or IV and oral glucocorticoids ($P = 0.18$). Patients who received glucocorticoids were, however, more likely to achieve an ACR20 response by week 4 than those who received no glucocorticoids. Consequently, since glucocorticoid treatment was not a prerequisite for the efficacy of rituximab, efficacy results are expressed using pooled glucocorticoid dose cohorts within each rituximab group.

The time course for achieving an ACR response showed early separation of rituximab from placebo. The proportion of patients who achieved an ACR20 response in the placebo group leveled off at ~30% at week 4, while in the rituximab groups, it continued to increase, leveling off at ~55% at 12 weeks. The same was essentially true for ACR50 and ACR70 responses, except in the rituximab 2 1,000-mg infusions group, in which ACR70 was seen in 10% of patients at week 12 and increased to 20% at week 24, suggesting a time-related effect on achievement of higher-level responses at this dose.

Changes in the DAS28 at week 24 reflected ACR response findings. Analysis of variance showed the adjusted mean change in the DAS28 from baseline to be significantly greater in patients treated with rituximab 2 500-mg and 2 1,000-mg infusions (−1.79 and −2.05, respectively) than in patients treated with placebo (−0.67; $P < 0.0001$). Similarly, EULAR responses in patients treated with rituximab at either dosage were significantly greater than those seen in patients who received placebo (Figure 2B). Compared with placebo, moderate or good EULAR responses were achieved in a significantly greater proportion of patients treated with rituximab 2 500-mg infusions ($P < 0.0001$) and rituximab 2 1,000-mg infusions ($P < 0.0001$), with a trend toward a higher proportion of patients achieving a good EULAR response with 2 1,000-mg infusions of rituximab.

CRP levels fell by a mean of 1.7 mg/dl between baseline and week 24 in patients treated with the active rituximab regimens. The corresponding mean change in CRP in the rituximab placebo group was 0.1 mg/dl. These differences in favor of the active rituximab groups were observed after week 4 and continued to week 24. In patients who were RF-positive at baseline, the proportion with RF titers below the level of detection by week 24 was 1.6% in the placebo group and 12% and 9% in the rituximab 2 500-mg infusions and 2 1,000-mg infu-

Samsung Bioepis Exhibit No. 1002
Page 7895

Table 3. Most frequently reported adverse events in the safety population (n = 465)*

Adverse events	Rituximab placebo (n = 149)	Rituximab 2 500-mg infusions (n = 124)	Rituximab 2 1,000-mg infusions (n = 192)
All events	105 (70)	100 (81)	164 (85)
Exacerbation of RA	44 (30)	21 (17)	27 (14)
Headache	19 (13)	14 (11)	21 (11)
Nausea	13 (9)	8 (6)	19 (10)
URI	9 (6)	10 (8)	12 (6)
Nasopharyngitis	8 (5)	7 (6)	10 (5)
Arthralgia	5 (3)	5 (4)	11 (6)
Diarrhea	8 (5)	7 (6)	6 (3)
Fatigue	8 (5)	5 (4)	8 (4)
Hypertension	4 (3)	5 (4)	12 (6)
Rigors	3 (2)	5 (4)	13 (7)
Dizziness	6 (4)	4 (3)	10 (5)
Serious noninfection adverse events	2 (1)†	9 (7)‡	9 (5)§
Serious infections	2 (1)	0	4 (2)

* All adverse events affecting ≥5% of patients in any one group are shown. Values are the number (%). RA = rheumatoid arthritis; URI = upper respiratory tract infection.
† Food allergy and exacerbation of RA in 1 patient each.
‡ Cerebral infarction, fatal cerebrovascular accident unrelated to study treatment, convulsion, myocardial infarction, supraventricular tachycardia, drug hypersensitivity (occurring within 24 hours of rituximab infusion), failure of implant, lower limb fracture, and interstitial lung disease in 1 patient each.
§ Epilepsy, serotonin syndrome, chest pain, generalized edema (occurring within 24 hours of rituximab infusion), exacerbation of RA, status asthmaticus, intestinal obstruction, metrorrhagia, and thromboangiitis obliterans in 1 patient each.

sions groups, respectively. This reflected a mean increase in total RF titers in the placebo groups (ranging from 7.1% to 37.4% among placebo groups) compared with a mean decrease in the active rituximab groups (ranging from 11.5% to 47.9% among rituximab groups).

Patient-reported outcomes mirrored the improvements in clinical and laboratory findings in the primary ITT population. Changes in the mean DI scores of the HAQ between baseline and week 24 were −0.43 and −0.49 in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively, compared with −0.16 in the rituximab placebo group. The proportions of patients with an improvement of −0.22 from baseline (minimum clinically important difference [MCID] [19]) were 63%, 67%, and 34%, respectively in these 3 groups. Furthermore, changes in fatigue, measured as the percentage improvement in the FACIT-F score between baseline and week 24, were 20% and 28% in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively, compared with 4% in the placebo group. In both rituximab groups, the mean change from baseline in the FACIT-F score exceeded 4 points, which is the MCID (16). This was not the case in the placebo group.

The inclusion of data from RF-negative patients in the analyses did not alter the primary outcome. In the combined RF-positive and RF-negative population, 52% of the rituximab 2 1,000-mg doses group achieved an ACR20 response at week 24 compared with 32% of the placebo group. An exploratory analysis of RF-negative patients alone proved inconclusive, since it was confounded by an unusually high placebo response (52% versus 48% in the rituximab group) and a relatively small sample size (21 and 63 patients, respectively).

There was a significant difference at week 24 between the absolute proportions of US and non-US patients who achieved an ACR20 response across treatment groups, including placebo ($P = 0.002$), with an ACR20 response being more frequent in the non-US region. Between regions, the efficacy of rituximab relative to placebo (i.e., the difference between placebo and active drug responses) was similar, and consequently, there was no significant interaction between rituximab and region ($P = 0.797$). An exploratory analysis of the 2 regions showed that the only major baseline difference between the US and non-US populations was the proportion of patients previously exposed to biologic therapy (55% of US versus 23% of non-US patients). While there was no identified interaction between prior use of biologic therapies and rituximab treatment, ACR20 response rates were generally lower in patients with prior exposure to biologic therapies.

Safety outcomes. Overall, 70% of patients treated with placebo reported at least 1 adverse event at any time during the study period, compared with 81% and 85% of patients in the rituximab 2 500-mg and 2

Samsung Bioepis Exhibit No. 1002
Page 7896

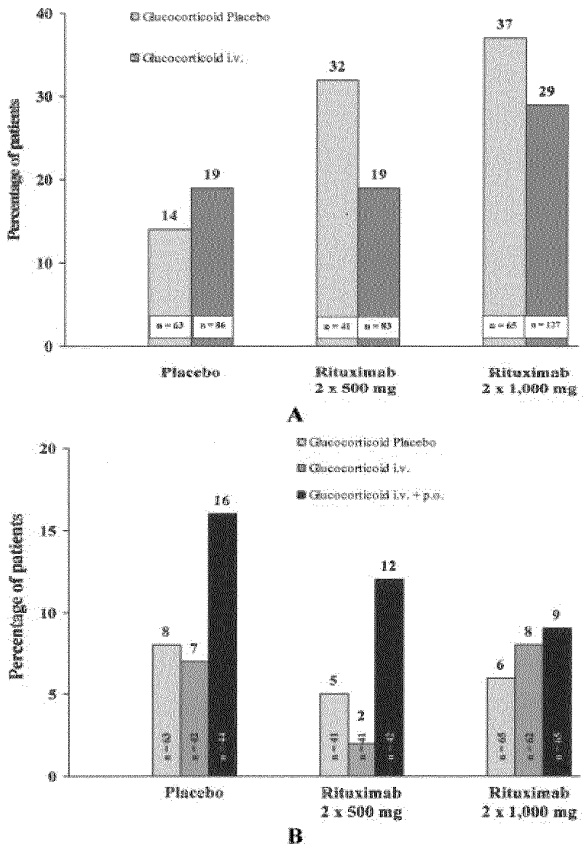


Figure 3. Effect of glucocorticoids on the frequency of acute infusion reactions after **A,** the first infusion and **B,** the second infusion. IV = intravenous; PO = by mouth.

1,000-mg infusions groups, respectively. The majority (82%) of events in each group were mild to moderate in severity (NCI CTC grades 1–2). A similar proportion of patients reported severe (NCI CTC grade 3) events in each group (18% of patients receiving placebo and 17% and 18% of patients in the rituximab 2 500-mg and 2 1,000-mg infusions groups, respectively). The most frequently reported adverse events in each treatment group are shown in Table 3 and were mainly associated with the first infusion. Exacerbation of RA, reported as an adverse event, distinguished rituximab placebo from both doses of active rituximab, reflecting the efficacy of rituximab relative to placebo. There were no unexpected adverse events in the RF-negative subgroup.

Infusion-associated events. Infusion-associated events (defined as any adverse event that occurred during or within 24 hours of rituximab infusion) occurred with the first infusion in 18% of patients who received placebo and in 31% and 38% of patients who received rituximab 2 500-mg and 2 1,000-mg infusions, respectively.

Acute infusion reactions. Symptoms or signs suggesting an acute infusion reaction (pruritus, fever, urticaria/rash, chills, pyrexia, rigors, sneezing, angioneurotic edema, throat irritation, cough and bronchospasm, hypotension, or hypertension) were more commonly associated with the first infusion of rituximab compared with placebo. Overall, acute infusion reactions were reported at the first infusion in 17% of placebo-treated patients compared with 23% and 32% of patients who received 2 500-mg or 2 1,000-mg rituximab, respectively. However, premedication with IV glucocorticoid significantly reduced both the incidence and severity of acute infusion reactions. Without glucocorticoid premedication, these events were reported by 14% of the placebo group, 32% of the rituximab 2 500-mg infusions group, and 37% of the rituximab 2 1,000-mg infusions group, as compared with 19%, 19%, and 29% of these groups, respectively, following IV glucocorticoid premedication (Figure 3A).

The number of patients who experienced these symptoms following the second infusion of the treatment course was substantially lower in all groups, irrespective of glucocorticoid premedication (Figure 3B). The addition of 2 weeks of oral prednisone treatment did not appear to provide any further reduction of acute infusion reactions with the second infusion.

Two adverse events occurred within the first 24 hours of the first infusion: 1 case of drug hypersensitivity reaction occurred in the rituximab 2 500-mg infusions group, and 1 case of generalized edema occurred in the rituximab 2 1,000-mg infusions group. Both events occurred in patients who had not received IV glucocorticoid premedication. Five patients (2.6%) in the 2 1,000-mg infusions rituximab group were withdrawn from treatment because of acute infusion-associated events following the first infusion. Four of these patients had not received glucocorticoid premedication.

Infection-related adverse events. Adverse events classed as infections and infestations were reported in 28% of the placebo group and in 35% of each of the active rituximab groups. The type and severity of infections were similar across the placebo and active rituximab groups (the most common being respiratory tract infections, urinary tract infections, and nasopharyngitis),

Samsung Bioepis Exhibit No. 1002
Page 7897

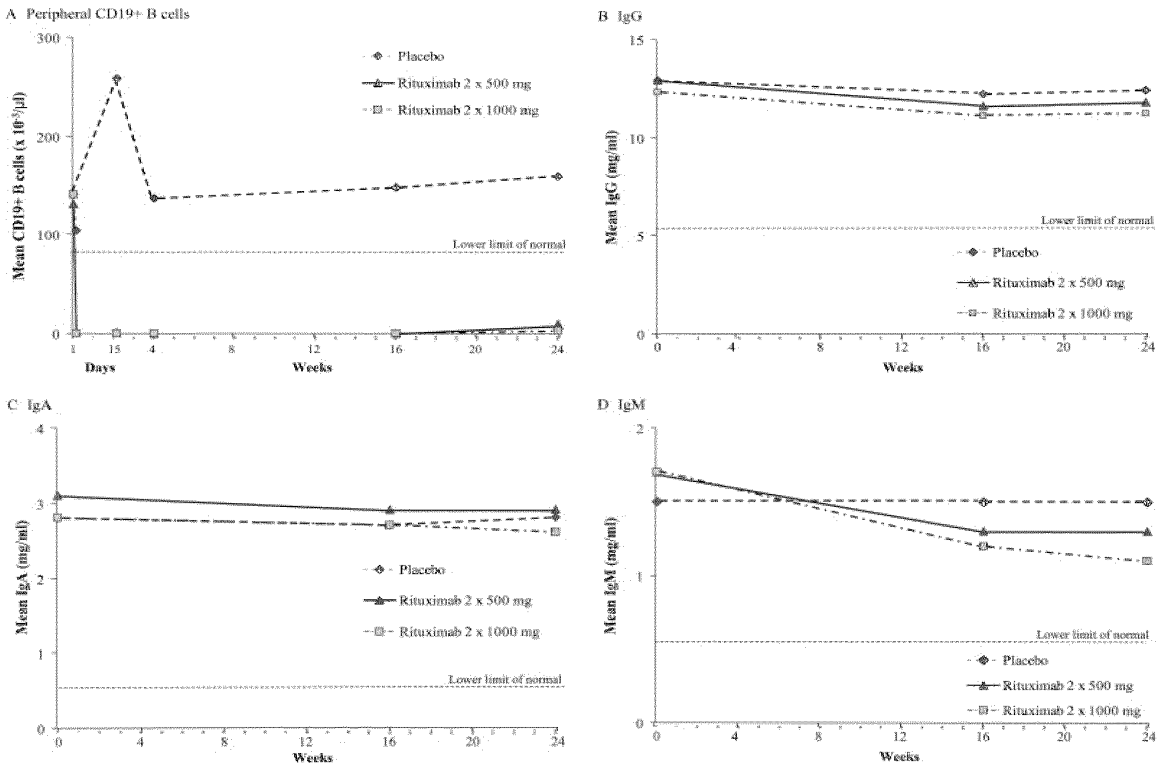


Figure 4. Levels of **A,** peripheral blood CD19+ B cells, **B,** IgG, **C,** IgA, and **D,** IgM during the 24-week treatment period.

so the pattern of infections did not distinguish between placebo- and rituximab-treated patients.

Six serious infections were reported. Two (1%)occurred in the placebo group (pneumonia and respiratory tract infection) and 4 (2%) in the rituximab 2 1,000-mg infusions group (2 cases of pyelonephritis, and 1 each of bronchitis and epiglottitis). These events resolved without sequelae. No serious infections occurred in the rituximab 2 500-mg infusions group. The rate of serious infections per 100 patient-years was 3.19 for the rituximab placebo group, 0 for the 2 500-mg infusions of rituximab group, and 4.74 for the 2 1,000-mg infusions of rituximab group.

Serious adverse events. A total of 26 serious adverse events were reported, 4 (3%) in the placebo group (3%), 9 in the rituximab 2 500-mg infusions group (7%), and 13 in the rituximab 2 1,000-mg infusions group (7%), with no consistent pattern or signal. Of these, 20 were noninfection events in 2, 9, and 9 patients in the placebo, rituximab 2 500-mg, and rituximab 2 1,000-mg infusions groups (1%, 7%, and 5%, respectively) (Table 3). Five events were nervous system disorders, while others were distributed evenly across body systems in no discernible pattern. There was 1 fatal event, which occurred in the rituximab 2 500-mg infusions group. A 73-year-old woman died of a cerebral infarction 167 days after the first infusion of rituximab. This patient had a history of atrial fibrillation, hypertension, diabetes mellitus, and hyperlipidemia; at the time of enrollment into the trial, she was receiving concomitant celecoxib, MTX, folic acid, lisinopril, atorvastatin, and sertraline.

Pharmacodynamics. In the placebo group, peripheral B cell levels remained stable around baseline levels throughout the study period, after an initial transient increase during the 2-week glucocorticoid dosing period between rituximab placebo infusions. Treatment with rituximab was associated with nearly complete

Samsung Bioepis Exhibit No. 1002
Page 7898

depletion of peripheral blood B cells, which persisted throughout the 24-week study period. Peripheral blood B cell recovery began after week 16 (Figure 4A).

Although peripheral blood B cell levels were depleted in rituximab-treated patients, mean and median Ig levels (IgG, IgA, and IgM) remained above the laboratory lower limit of normal at all times; the greatest change from baseline occurred in IgM (Figures 4B–D). Anti-tetanus titers remained stable.

Antibodies to the test agent, i.e., HACA (analyzed up to and including week 24), were detectable in 0.7%, 4.2%, and 2.7% of the placebo, rituximab 2 500-mg, and rituximab 2 1,000-mg infusions groups, respectively. There were no serious adverse events in the group of patients with HACAs. Moreover, 6 of the 10 rituximab-treated patients with positive HACAs at week 24 achieved ACR20 responses at that time.

DISCUSSION

Results of the DANCER trial have confirmed that a single course of rituximab, given as 2 infusions 2 weeks apart, is highly effective over 24 weeks in the treatment of active RA in patients who have shown an incomplete response to MTX. Followup of these patients beyond the primary end point is ongoing, and radiologic outcomes (not measured in this study) will be addressed in future studies. Rituximab has a novel mode of action that results in the depletion of B cells, and it is therefore distinct from other biologic therapies for RA that target T cells and their related cytokines. The present study corroborates earlier studies that support a role for B cells in the inflammatory processes that underlie RA (9,10,20–24).

Both rituximab dosages were effective, with 54–55% of patients achieving an ACR20 response by week 24, and a high proportion achieving ACR50 or ACR70 responses. Importantly, clinical improvements in rituximab-treated patients were reflected in patient- and physician-reported outcome measures, as well as in changes in laboratory parameters. Moreover, the beneficial effects of rituximab were often evident early in the course of treatment.

In general, the efficacy results from the DANCER study broadly mirror those from a phase IIa study of rituximab in 161 RF-positive RA patients (9), although in the latter, a higher ACR20 response rate was observed. A possible explanation for this discrepancy is the prior use of biologic therapies at baseline (an indication of refractory disease). In the phase IIa study, only ~10% of patients had been exposed to a biologic agent, whereas in the DANCER trial, the proportion was 30–40% in the active rituximab groups. A subanalysis of DANCER trial patients who received active rituximab with no prior exposure to biologic therapies determined an ACR20 response rate of 62% for this group, which is closer to previously reported rates (9). Previous use of biologic agents may also be a contributory factor to the lower absolute ACR response observed in US patients compared with non-US patients in this study. A greater proportion of US patients had received such treatment and may therefore have constituted a more refractory population. Nevertheless, rituximab was similarly effective relative to placebo both in patients who had received prior biologics and those naive to these therapies.

No dose-response relationship was established, based on the ACR20 criteria, for the 2 rituximab doses studied. There were, however, trends to indicate that the dose may influence the achievement of high-level response (i.e., ACR70 response and good EULAR response), and further evaluation of both rituximab doses is warranted.

The use of concomitant glucocorticoids with rituximab was based on the successful application of similar regimens in patients with NHL (10) and the initial trials in patients with RA (9,20,21). This trial has, however, established that glucocorticoids play no significant role in determining the primary end point of an ACR20 response at 24 weeks in RA patients treated with rituximab, although they may enhance the early response (up to 4 weeks) by virtue of their antiinflammatory properties. Consequently, the use of glucocorticoids does not appear to be a prerequisite for a clinical response to rituximab.

Both dosages of rituximab were generally well tolerated, with a low incidence of serious adverse events, including serious infections. The majority of adverse events were infusion associated, mild to moderate in intensity, and easily managed. Premedication with IV glucocorticoids did, however, reduce the incidence and severity of infusion-associated events following the first infusion of rituximab. The incidence of reactions following the second infusion was low in all groups, with 2 weeks of oral glucocorticoid treatment providing no additional safety benefit over IV glucocorticoid premedication alone. Overall, the incidence of infusion-related adverse events in RA patients appears to be lower than that observed in NHL patients (who may be prone to such events because of elevated levels of white blood cells or tumor lysis) (10,25).

All patients had depleted B cell levels following rituximab treatment, with peripheral blood B cell levels falling to below the lower limit of normal. Mean and

Samsung Bioepis Exhibit No. 1002
Page 7899

median Ig levels stayed within the normal range, but there was a downward trend specifically with IgM; however, there was no significant change in acquired immunity. Overall, the incidence of immunogenicity (HACAs) was low and had no apparent clinical relevance over the primary 24-week course of this trial, but patients continue to be monitored for this, together with all other pharmacodynamic parameters, including CD19 and Ig levels.

The DANCER study is a novel design for the study of RA: 9 treatment groups with a logistic regression main-effects model as its primary means of analysis. The complexity of the study was driven by a need to understand the effects of different components of a potential therapeutic regimen (i.e., rituximab dose and effects of glucocorticoids). Both objectives were met, but several factors must be taken into account when interpreting the results. First, extrapolation of these data to the larger RA population must be done with care, given the relatively small number of patients involved. In particular, specific comparison between any of the individual 9 groups is inappropriate; however, valuable guidance concerning both the use of glucocorticoids and the dosage of rituximab has been obtained. Second, it should be acknowledged that the present data represent just 24 weeks of followup; longer-term followup will be required to better characterize safety and, possibly, to identify potential differences between rituximab dosages. Last, with regard to the subset of RF-negative patients, small numbers of patients and a large placebo effect prevent the drawing of firm conclusions about the safety and efficacy of rituximab in this population.

In conclusion, both dosages of rituximab were highly effective and generally well tolerated when given with continuing MTX to patients with active RA. The study has also shown that concomitant glucocorticoids are not a prerequisite for the efficacy of rituximab in RA. However, premedication with IV glucocorticoids does appear to ameliorate acute infusion reactions. Both dosages of rituximab explored in this study warrant further differential exploration and longer-term followup.

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APPENDIX A: MEMBERS OF THE DANCER STUDY GROUP

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Page 7901

D46

NATURAL HISTORY OF PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

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Abstract *Background.* Paroxysmal nocturnal hemoglobinuria (PNH), which is characterized by intravascular hemolysis and venous thrombosis, is an acquired clonal disorder associated with a somatic mutation in a totipotent hematopoietic stem cell. An understanding of the natural history of PNH is essential to improve therapy.

Methods. We have followed a group of 80 consecutive patients with PNH who were referred to Hammersmith Hospital, London, between 1940 and 1970. They were treated with supportive measures, such as oral anticoagulant therapy after established thromboses, and transfusions.

Results. The median age of the patients at the time of diagnosis was 42 years (range, 16 to 75), and the median survival after diagnosis was 10 years, with 22 patients (28 percent) surviving for 25 years. Sixty patients have died; 28 of the 48 patients for whom the cause of death is known died from either venous thrombosis or hemorrhage. Thirty-one patients (39 percent) had one or more episodes of venous thrombosis during their illness. Of the 35 patients who survived for 10 years or more, 12 had a spontaneous clinical recovery. No PNH-affected cells were found among the erythrocytes or neutrophils of the patients in prolonged remission, but a few PNH-affected lymphocytes were detectable in three of the four patients tested. Leukemia did not develop in any of the patients.

Conclusions. PNH is a chronic disorder that curtails life. A spontaneous long-term remission can occur, which must be taken into account when considering potentially dangerous treatments, such as bone marrow transplantation. Platelet transfusions should be given, as appropriate, and long-term anticoagulation therapy should be considered for all patients. (N Engl J Med 1995;333:1253-8.)

PAROXYSMAL nocturnal hemoglobinuria (PNH) is an acquired disorder of hematopoiesis characterized by intravascular hemolysis and manifested by episodes of hemoglobinuria and life-threatening venous thromboses.[1] The cellular abnormality in this disorder is caused by a somatic mutation in a totipotent hematopoietic stem cell.[2,3] The cells derived from the abnormal clone are deficient in all surface proteins normally attached to the cell membrane by a glycosylphosphatidylinositol anchor.[4,5]

PNH was first described as a distinct clinical entity in 1882.[6] The cardinal diagnostic test, introduced in the late 1930s, is Ham's test,[7] which is based on the increased sensitivity of PNH-affected erythrocytes to lysis by complement. Deficiency of an antigen known as the membrane inhibitor of reactive lysis (CD59) is largely responsible for the hemolysis[8,9] and is implicated in the tendency for patients to have thromboses.[9] In the past two years the biochemical defect underlying PNH has been pinpointed at an early step in the biosynthesis of glycosylphosphatidylinositol molecules — namely, the transfer of *N*-acetylglucosamine to phosphatidylinositol.[10-12] The protein required for this step is encoded by a gene, *PIG-A*, that is somatically mutated in patients with PNH.[13-17]

Despite the remarkable progress in our understanding of this disorder, treatment has remained largely supportive. The only potentially curative therapy currently available is bone marrow transplantation,[18,19] which is associated with substantial morbidity and mortality.[20] To determine whether the management of PNH can be improved, it is important to know its natural history. We report the results of a long-term study (up to 48 years after the diagnosis) of a group of 80 patients with PNH seen at one institution between 1940 and 1970. This study has uncovered some surprising information on the natural history of the disorder and has identified relatively simple therapies that may reduce the associated morbidity and mortality.

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METHODS

Patients

Eighty patients with PNH were referred to Hammersmith Hospital in London between 1940 and 1970. In all patients the diagnosis was established or confirmed by a positive Ham's test.[21] This group of patients was last described in 1972.[22] Follow-up data have been obtained by contacting the patients' primary physicians and reviewing death certificates.

Complications and Causes of Death

The complications and causes of death reported were either unequivocally diagnosable on clinical grounds or were diagnosed after death. If a complication was not confirmed, it is not reported here. Thus, the reported incidence of complications is likely to be an underestimate, because many of the patients were seen at Hammersmith Hospital only infrequently. We compared the survival of the patients with the survival of a control group matched for sex and age.

Analysis of Blood Samples

In 1993 venous blood samples were obtained from five patients in either acid–citrate–dextrose or EDTA for the performance of Ham's tests and flow cytometric studies of the expression of glycosylphosphatidylinositol-linked proteins on erythrocytes and white cells, as previously described.[23,24] By definition, normal cells express normal levels of glycosylphosphatidylinositol-linked proteins, whereas PNH-affected cells are deficient in all these proteins.

RESULTS

Follow-up Studies

Sixty of the 80 patients were known to have died, and 6 patients were lost to follow-up before 1972. The remaining 14 patients were alive when last seen between

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Page 7902

1975 and 1994, and 9 of the 14 were known to be alive in 1994.

Clinical Presentation

The presenting features or initial diagnoses were reported previously.[22] The median age at the time of diagnosis was 42 years (range, 16 to 75). Forty-three (84 percent) of the 51 patients for whom reliable information was available had episodes of hemoglobinuria as a chief symptom at some time during their illness.

Course of Illness and Survival

The course of the disease in each of the 80 patients is shown in Figure 1. From the actuarial survival curve for the group, we calculated a median survival of approximately 10 years (Fig. 2). Twenty-five years after the diagnosis, 58 patients (72 percent) had died, and 22 (28 percent) were alive. The median age at the time of death was 56 years (range, 20 to 84), with a median interval of 10 years (range, 0 to 48) between diagnosis and death. Patient 10 received the diagnosis of PNH in 1939, had a spontaneous remission in 1949, and died of bronchial carcinoma in 1987, 48 years after the diagnosis.

Cause of Death

For 12 of the 60 patients who died (Table 1), the cause of death was unknown. In 28 of the other 48 patients (58 percent), the cause of death (either thrombosis or hemorrhage attributable to thrombocytopenia) was directly related to PNH. In the other 20 patients (42 percent), the cause of death was unrelated to PNH.

Venous Thrombosis

Thirty-one patients (39 percent) were known to have had one or more episodes of venous thrombosis during the course of their illness (Table 2), and several patients had repeated thromboses. In many patients the thrombosis was either fatal or life-threatening, and it was often unheralded.

Spontaneous Remission

Twelve patients (15 percent) had spontaneous clinical remissions (Fig. 1), with negative Ham's tests in all nine who were tested. We later obtained blood samples from five of these nine patients; in all five the erythrocytes expressed normal levels of glycosylphosphatidylinositol-linked proteins, with no demonstrable PNH-affected erythrocytes (Fig. 3A). In addition, of the four patients tested, none had PNH-affected neutrophils (Fig. 3A), but three of the four had small numbers of PNH-affected lymphocytes (Fig. 3B). Further analysis by double fluorescent staining demonstrated a subpopulation of PNH-affected lymphocytes among CD4+

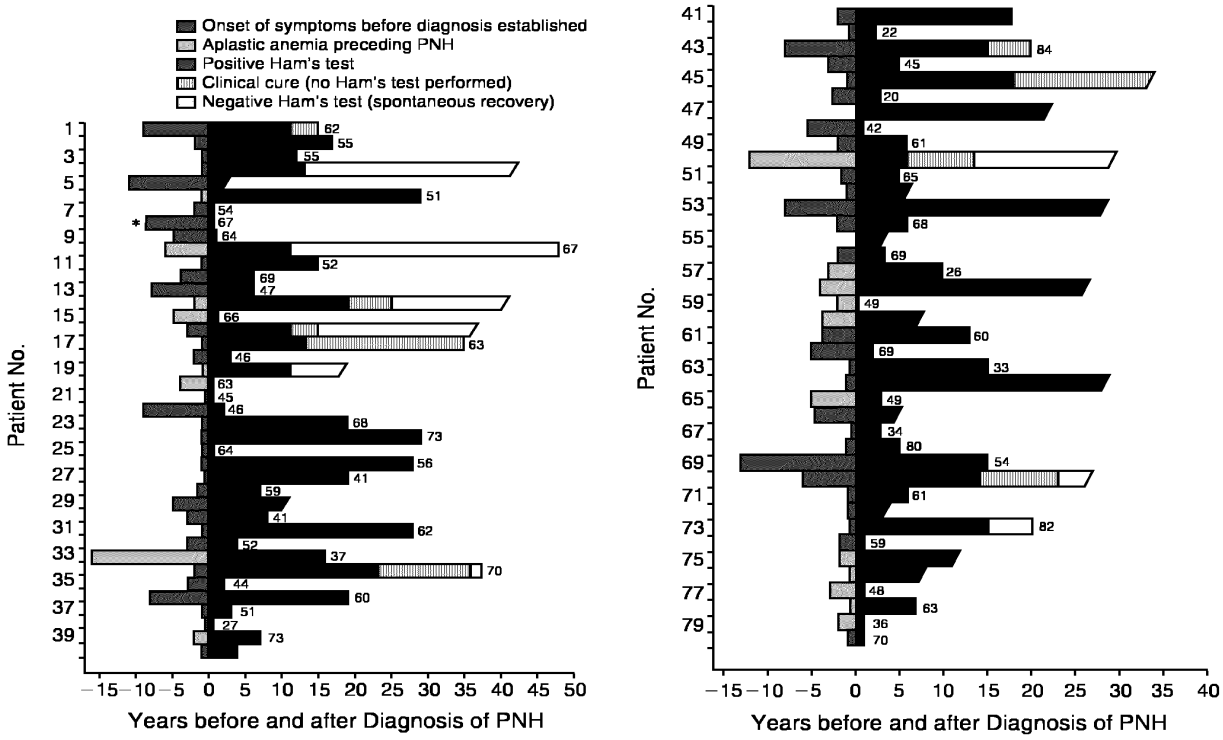


Figure 1. Course of Illness in 80 Consecutive Patients with PNH.

The x axis indicates the years before and after the diagnosis of PNH, which is denoted by the zero on the axis. Bars ending in a straight line indicate patients who died, and their ages at death are shown; bars ending in a diagonal line indicate surviving patients. Nine patients had complete clinical recovery, with negative Ham's tests. Three additional patients had protracted clinical remissions, but Ham's test was not repeated during remission. The asterisk indicates a prolonged but unspecified period of symptoms before the diagnosis was established.

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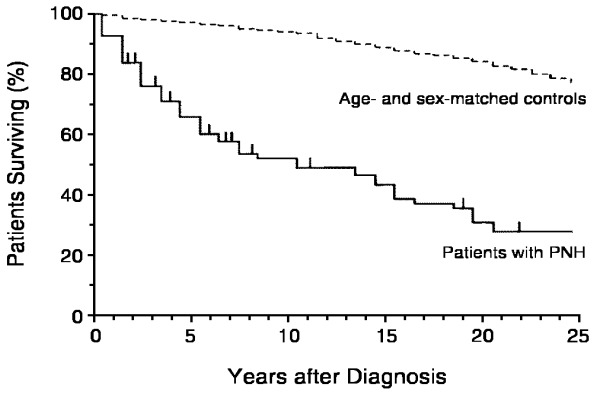


Figure 2. Actuarial Survival from the Time of Diagnosis in 80 Patients with PNH.

The median survival was 10 years. The expected survival of an age- and sex-matched control group is shown for comparison.

T cells, CD8+ T cells, and CD19+ B cells in all three patients.

Associated Disorders

Twenty-three patients (29 percent) received a diagnosis of aplastic anemia before the diagnosis of PNH. Nine of these patients subsequently had hemolytic PNH, with episodes of overt hemoglobinuria, and six did not; whether the remaining eight patients ever had hemoglobinuria could not be reliably determined.

At the time of the diagnosis, 64 of the 80 patients (80 percent) had thrombocytopenia (platelet count, <150,000 per cubic millimeter), and 38 patients (48 percent) had severe thrombocytopenia (platelet count, <50,000 per cubic millimeter). In addition, 44 patients (55 percent) had neutropenia (neutrophil count, <1500 per cubic millimeter).

In five patients the disorder progressed from hemolytic PNH to aplasia. Ham's test remained positive in four of these patients, but the fifth (Patient 34) subsequently had a negative Ham's test, with no PNH-affected erythrocytes or neutrophils. The patient died from bleeding due to severe thrombocytopenia 37 years after the diagnosis of PNH and at least 8 years after Ham's test had become negative.

Leukemia was not known to have developed in any of the patients.

Discussion

Clinical Features

In this group of 80 patients who were followed for up to 48 years, the median actuarial survival was 10 years, with 28 percent of the patients surviving for 25 years. Death was directly attributable to PNH or to bone marrow hypoplasia in 58 percent of the patients who died. Therapy with platelet concentrates should reduce mortality from hemorrhage due to thrombocytopenia.

At least 39 percent of the patients had venous thrombosis at some time during their illness. Many patients had initial thromboses that were life-threatening, but two (Patients 17 and 70), who probably had postpartum hepatic-vein thrombosis, recovered and survived for 34 and 27 years, respectively, after the thrombosis. One other patient (Patient 34) survived for 24 years after a thrombosis of the inferior mesenteric vein.

Spontaneous Remission

In some patients with PNH, the severity of the illness diminishes with time,[25] and some patients have a complete clinical remission,[22,26] although laboratory abnormalities may persist for years.[27] In this series 15 percent of the patients had spontaneous clinical remissions, and Ham's test became negative in the patients who were tested. Neither the severity of symptoms or complications nor the proportion of red cells lysed in Ham's test was correlated with an eventual remission. Among the patients with remissions, one (Patient 14) had severe, transfusion-dependent PNH and thromboses, and at

Table 1. Causes of Death in 60 Patients with PNH.

Cause of Death	No. of Patients
Probably related to PNH	
Venous thrombosis	
Hepatic vein	7
Inferior vena cava	1
Cerebral vein	1
Mesenteric vein	2
Pulmonary embolism	3
Hemorrhage	
Gastrointestinal	6
Subarachnoid	3
Intracerebral	2
Miscellaneous	
Liver failure	2
Intraabdominal event	1
Probably unrelated to PNH	
Arterial thrombosis	
Myocardial infarction	6
Cerebrovascular accident	2
Bronchopneumonia	3
Chronic obstructive airways disease plus cor pulmonale	2
Cardiac tamponade	1
Constrictive pericarditis	1
Renal failure	1
Amyloidosis	1
Lymphoma	1
Bronchial carcinoma	1
Epilepsy	1
Unknown	12

Table 2. Sites and Types of Thrombosis.

Site and Type of Thrombosis	No. of Patients
Intraabdominal	
Hepatic vein	8
Inferior vena cava	3
Mesenteric vein	4
Splenic vein	1
Renal vein	1
Unspecified	1
Other venous sites	
Cerebral vein	4
Pulmonary embolism	9
Deep vein	7
Superficial	3
Arterial	
Myocardial infarction	6
Cerebrovascular accident	2

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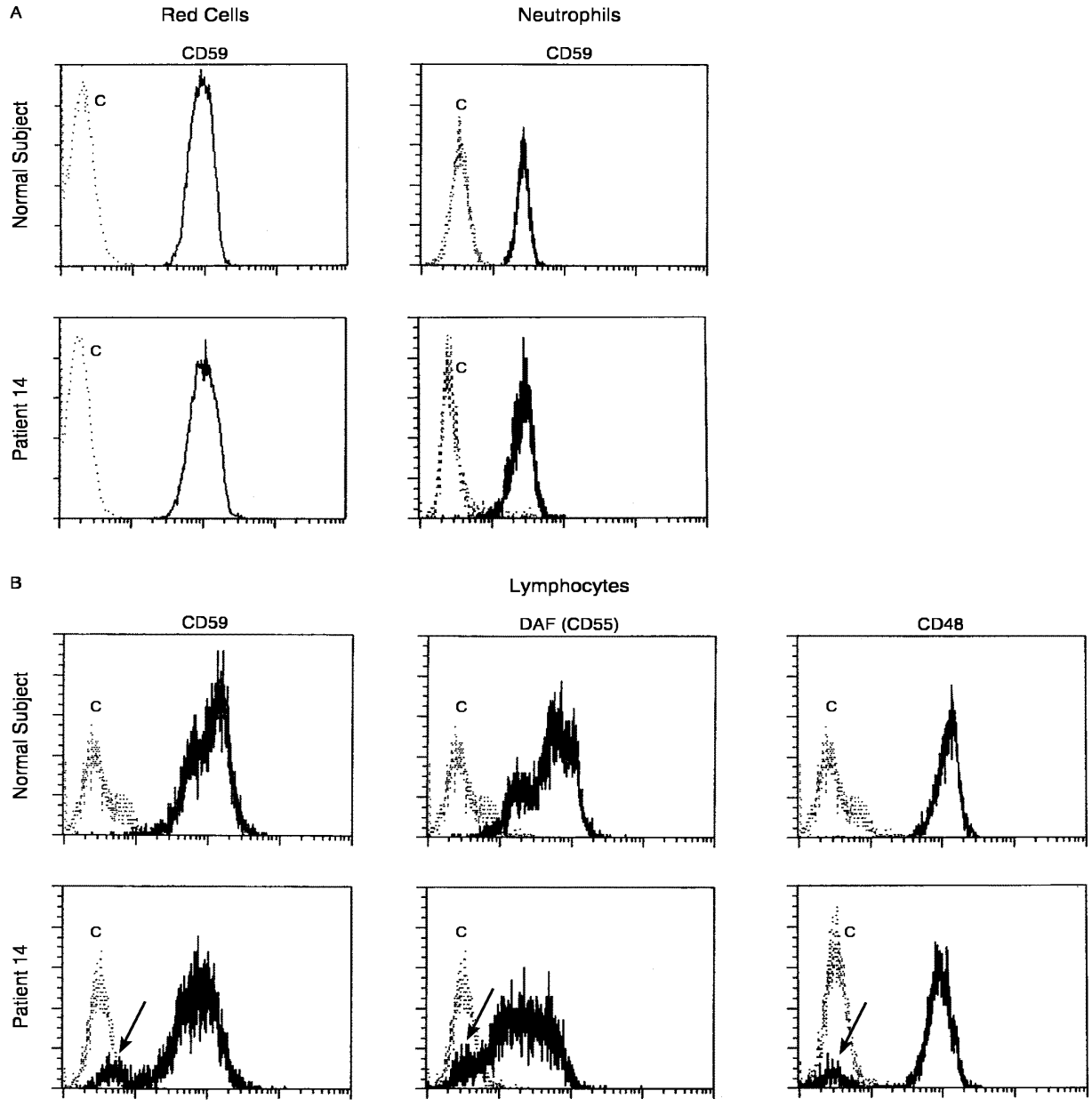


Figure 3. Flow-Cytometric Analysis of Blood Cells from a Normal Subject and Patient 14 after Spontaneous Recovery from PNH.

The analysis was carried out 20 years after the patient's clinical remission began and 17 years after her Ham's test had become negative. Panel A shows a unimodal distribution of cells with normal reactivity after reaction with anti-CD59 antibody (a molecule absent from the surface of PNH-affected cells). The distributions obtained with the patient's red cells and neutrophils are indistinguishable from those of the cells from a normal subject, demonstrating that no PNH-affected cells remain. C indicates distributions obtained in control experiments with an antibody directed against a molecule absent from normal blood cells.

Panel B shows the results of an analysis, similar to that in Panel A, carried out on peripheral-blood lymphocytes. The distributions of cells from a normal subject show that, as compared with the results of analyses with negative control antibody (C), all the lymphocytes reacted with all three antibodies used. The distribution is unimodal for CD48 and bimodal for CD59 and CD55. In contrast, the cells from Patient 14 have a small population of lymphocytes that are unreactive with all three antibodies (arrows), which indicates that these cells lack the corresponding antigen protein molecules on their surfaces. The fact that there is a peak of antigen-negative cells for three different proteins that are normally attached to the cell membrane by a glycosylphosphatidylinositol anchor means that the anchor is absent, which is characteristic of the PNH phenotype. The finding that the PNH clone was still present when there were no PNH-affected erythroid or myeloid cells in Patient 14 during a clinical and hematologic remission is consistent with the notion that this clone does not have a selective advantage once normal hematopoiesis has resumed. DAF denotes decay-accelerating factor.

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least two had over 50 percent lysis in earlier Ham's tests, suggesting that they had few residual normal stem cells at the height of their illness. The spontaneous remissions occurred between 10 and 20 years after the diagnosis of PNH. Of the 35 patients who survived 10 years or more, 12 (34 percent) eventually had remissions. A possible explanation for spontaneous remission is that the clones of PNH-affected cells have a finite life span, like normal somatic cells. Recovery may thus depend on the presence of normal stem cells capable of repopulating the bone marrow.[28]

There were no erythrocytes or neutrophils affected by PNH after recovery, but three of four patients tested had some residual abnormal lymphocytes. These results are consistent with the fact that lymphocytes have a much longer life span than myeloid and erythroid cells and that committed lymphocyte progenitor cells undergo division less frequently than the other cells. If the aging process postulated as being responsible for the gradual disappearance of clones of PNH-affected cells is related to the number of cell divisions, this may explain why abnormal lymphocytes persist for many years after the disappearance of abnormal neutrophils and erythrocytes.

PNH and Bone Marrow Failure

The data on this series of patients provide further evidence of the close relation between PNH and aplastic anemia. The coexistence of the two conditions in one patient was first reported in 1944,[29] with subsequent reports in 1952[30] and 1961.[31] Cultures of peripheral blood and bone marrow from patients with PNH consistently show reduced numbers of erythroid progenitor cells (erythroid burst-forming units) and myeloid progenitor cells (granulocyte–macrophage colony-forming units), even in the absence of pancytopenia,[32,33] as in patients with aplastic anemia.[34] In addition, PNH eventually develops in 10 to 31 percent of patients with aplastic anemia treated with immunosuppressive agents.[35] It is important to emphasize that PNH is not a complication of immunosuppressive treatment. Instead, the majority of patients with this disorder probably have an underlying aplastic process. Clones of PNH-affected cells may thus have a relative growth or survival advantage over the residual normal bone marrow in patients who have aplastic anemia.[1,28,36] It is conceivable that the mutation leading to the development of PNH occurs quite frequently but that, in the absence of marrow hypoplasia, the clone has difficulty establishing itself.[28]

PNH and Leukemia

Because of the reports of myelodysplasia and leukemia[1] — invariably, acute myeloid leukemia — in patients with PNH, it has been regarded as a preleukemic condition. Leukemia did not develop in any of our 80 patients, however, indicating that it is relatively rare in unselected patients with PNH. Acute myeloid leukemia eventually develops in approximately 5 percent of patients with aplastic anemia who survive the marrow aplasia.[35] Thus, aplastic anemia may predispose patients to both PNH and acute myeloid leukemia, but the development of PNH may not add to the risk of leukemia associated with uncomplicated aplastic anemia.

In most reported cases of leukemia in patients with PNH in whom the leukemic cells were studied,[37,38] the leukemic clone was derived from the PNH clone. According to a recent report of a patient with PNH who subsequently had a myelodysplastic syndrome, the blasts expressed normal levels of glycosylphosphatidylinositol-linked proteins,[39] indicating that the leukemic event may occur in the residual normal stem cells.

Management

The natural history of PNH is an important factor in decisions about therapy for individual patients. Thrombolytic treatment of hepatic-vein thrombosis with tissue plasminogen activator has been reported to be effective.[40] Oral anticoagulation therapy after venous thrombosis has been used since the 1950s.[25] In view of the high incidence of potentially fatal thrombosis, a strong case can be made for prophylactic anticoagulation treatment in all patients with PNH in whom there is no contraindication (such as severe thrombocytopenia). This approach may improve survival and reduce morbidity.

The only curative therapy at present is bone marrow transplantation, but it is available to only a small proportion of patients and is associated with substantial morbidity and mortality. The recent advances in the understanding of the pathogenesis of PNH and, in particular, of the molecular lesion open opportunities to explore new treatments, such as gene therapy. The possibility of spontaneous remission and of long-term survival must be taken into account when considering either bone marrow transplantation or other new treatments for this disorder.

We are indebted to the late Miss Eleanor Lloyd for her assistance in this project, to the patients, and to the physicians who originally referred the patients and provided follow-up data.

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IMAGES IN CLINICAL MEDICINE

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Samsung Bioepis Exhibit No. 1002
Page 7907

J A • K E M P

21 November 2018

Our Ref: C411599EP

SUBMISSION UNDER R116

Patent No:	2359834
Application No:	11001632.6
Patentee:	Alexion Pharmaceuticals, Inc.
Opponents:	(1) Amgen Inc. (2) Hoffmann Eitle

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CONTENTS

1 REQUESTS 1

2 DOCUMENTS 1

3 DECLARATION OF DR KLIBANOV AND INVENTIVE STEP 2

4 INSUFFICIENCY 4

5 AUXILIARY REQUESTS 5

6 SUMMARY 5

Samsung Bioepis Exhibit No. 1002
Page 7909

1 REQUESTS

1.1 The Patent Proprietor requests that the Opposition Division maintains the Patent as granted.

1.2 If the Opposition Division is not able to grant the Main Request, even after discussion at Oral Proceedings, then the Patent Proprietor requests that the patent is maintained on the basis of one of Auxiliary Requests 1 or 2 enclosed herewith. Also enclosed, to assist the Opposition Division, are tracked copies of Auxiliary Requests 1 and 2 showing the amendments which have been made compared with the Main Request.

1.3 It is further requested that, should any minor alterations be required to these Auxiliary Requests, an opportunity be provided to make such amendments at the Oral Proceedings.

1.4 We shall speak in English at the Oral Proceedings. We shall require interpretation into English should any other party speak in a language other than English.

1.5 We shall be accompanied at the hearing by Todd Spalding and Tara Rahemba of Alexion Pharmaceuticals, Inc and Dr Dino Miano, also of Alexion Pharmaceuticals, Inc and author of declaration D33. I request permission for Dr Miano to speak at the oral proceedings, should this be appropriate. Dr Miano's qualifications are set out in D33. Dr Miano may speak in relation to the commercial product, Soliris. Dr Miano may also discuss the subject matter contained in his declaration, in particular the stability data presented therein, as well as the content of Dr Treuheit's declaration D23.

2 DOCUMENTS

2.1 The following additional documents are cited and enclosed herewith:

D39 Declaration of Dr Alexander Klibanov.

D40 Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8.

D41 Alberts *et al*, Molecular Biology of the Cell, Third Edition, 1994, page 585.

2.2 We also enclose a consolidated list of all the documents cited by both Opponents and the Patent Proprietor, including the additional documents mentioned above.

Samsung Bioepis Exhibit No. 1002
Page 7910

3 Declaration of Dr Klibanov and Inventive Step

3.1 Dr Klibanov is an expert in the formulation of therapeutic proteins, including antibodies. In his declaration, Dr Klibanov provides his expertise on the issues which the skilled person in this field would have faced around 2007, the filing date of the present patent. Dr Klibanov particularly comments on the complexities and challenges which the skilled person would have faced in 2007, when developing a therapeutic antibody formulation.

3.2 In the Summons, the Opposition Division summarise the arguments of the Patent Proprietor in the submission of 22 March 2018 and comment briefly on the Proprietor's position that formulating antibodies was a complex area. It is the Proprietor's position that the difficulties in antibody formulation in 2007 cannot be overestimated and it is considered important that the significance of these issues is fully appreciated. Accordingly, Dr Klibanov, who is an expert in this field and was working in this area in 2007, has been asked to provide his expert opinion.

3.3 The relevance of Dr Klibanov's declaration to the Patent Proprietor's position on inventive step will be explained further below with reference to the problem and solution approach:

3.4 The Opposition Division set out their view that D1 can be taken as the closest prior art document. The difference between the claimed subject matter and that of D1 is at least the following:

- The formulation is a single use 300mg dosage form;
- The concentration is 10 mg/ml;
- The volume is 30ml;
- The solution is preservative free.

3.5 As explained in detail in section 5.3 of the 22 March 2018 submission, the technical effect of the claimed composition is extremely good long term stability, such that the antibody maintains its integrity and biological activity in the composition. Further, the 10 mg/ml concentration provides this enhanced stability in a low-volume liquid formulation which is easy for the clinician to store and use.

3.6 Accordingly, the objective technical problem to be solved over D1 is to provide a composition of eculizumab, an antibody having a unique, engineered structure, which provides the ideal and necessary balance of properties for use in the treatment of PNH, including enhanced maintenance of biological integrity following prolonged storage, whilst also providing a low-volume liquid formulation.

Samsung Bioepis Exhibit No. 1002
Page 7911

3.7 The solution to this problem is the 300 mg single-use dosage form of eculizumab, comprising 30 ml of a 10 mg/ml preservative free solution.

3.8 The skilled person could not have predicted, at the filing date of the patent, that the 10 mg/ml composition would provide the required balance of low volume formulation combined with high stability. Indeed, as Dr Klibanov explains in his declaration at paragraph 4, achieving long term stability was a major challenge at the time:

"One of the major challenges in developing a successful pharmaceutical formulation of a therapeutic antibody (and of any therapeutic protein) around 2007 was, and still is today, being able to create a composition that is stable enough for sufficiently long periods of time such that the antibody does not undergo deleterious physical or chemical degradation reactions or form aggregates prior to administration."

3.9 Dr Klibanov comments that a skilled person faced with the task of formulating a new therapeutic protein would not have expected to be able to develop a liquid composition at all, due to these potential instabilities (see paragraph 6, D39). Dr Klibanov goes on to discuss the difficulties of balancing the various causes of protein instability, concluding in paragraph 10:

"Given the complexity and the sheer number of the various modes of protein instability and the diverse mechanisms underlying each of them, there was no universal approach to successfully formulating an antibody in 2007 (nor is there one now). My both academic and industrial experience at the time was that the field of antibody formulation was difficult, case-specific, and uncertain. For these reasons, a person skilled in the art aiming to develop a liquid formulation of a new antibody with a sufficient storage stability at a high concentration would not have been able to predict which, if any, such formulation would meet the necessary requirements."

3.10 Dr Klibanov's view therefore confirms the unpredictability of the field. The Opponents suggest that the composition of eculizumab described in claim 1 would have been obvious, based on disclosures relating to formulations of different antibodies, in particular D20 and D21. Dr Klibanov's experience, however, demonstrates that this is not the case: as he confirms, in fact, each antibody needs to be considered individually. This requires a uniquely-designed, detailed research program to determine the ideal composition for a given antibody. A person skilled in the art would not have had any confidence that applying a formulation found to be successful for a different antibody would also be successful with eculizumab.

3.11 In particular, in paragraphs 8 and 9 of his declaration, Dr Klibanov explains that each of D20 and D21 referred to by the Opponents highlight the uniqueness of every antibody and the unpredictable nature of formulating a stable antibody composition. D20 teaches that *"each antibody seems to have a unique personality related to its requirements for stability"* (see

Samsung Bioepis Exhibit No. 1002
Page 7912

paragraph 9 of D39), and D21 stresses *"that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the difference in their sequence"* (see paragraph 9 of D39).

3.12 Further, eculizumab itself would have presented particular difficulties to the skilled person in developing a suitable formulation. As set out in paragraph 3.6 above, the objective technical problem relates to providing a suitable formulation for eculizumab, which has a unique, engineered structure. Dr Klibanov explains at paragraphs 7 to 9 of D39 that even small structural differences between antibodies can lead to significant differences in behaviour in liquid compositions. For instance, at paragraph 7, Dr Klibanov refers to a 2002 text book:

"*The book explains that 'the exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation' and that '[e]ven for closely related proteins, the relative stability and major pathways for degradation might be quite different.' (Ex. 2 at 185-186.) In the 2007 time-frame, researchers like myself understood that therapeutic antibody (or protein in general) formulations, especially liquid ones, are* ***not*** *amenable to a 'one size fits all' approach."*

3.13 The sensitivity of protein stability to primary sequence, which Dr Klibanov here refers to, would have been particularly relevant to eculizumab, given its uniquely-engineered structure.

3.14 Dr Klibanov's declaration therefore demonstrates that the skilled person in the art in 2007, faced with the issue of developing a pharmaceutical composition for eculizumab which provides the right balance of a low volume formulation with high stability, would not have been able to predict whether a liquid composition would be possible at all, let alone be able to predict, with a reasonable expectation of success, that a 10 mg/ml formulation in particular would provide the required balance of properties. For these reasons, the Proprietor maintains that the claimed subject matter is in accordance with Article 56 EPC.

4 Insufficiency

4.1 The skilled person would have had no difficulty in carrying out the invention as claimed, which is to provide a single-use dosage form of a sterile and preservative free solution comprising 30ml of a 10mg eculizumab/ml solution and to use it to treat PNH patients. This is because the full heavy and light chain sequences of eculizumab are disclosed in SEQ ID NOs: 2 and 4 in the patent, enabling the skilled person to produce eculizumab.

4.2 Although SEQ ID NO: 4 in the patent is the *immature* light chain sequence of eculizumab and thus contains the signal peptide, the signal peptide would be cleaved off during antibody production in the cell, and this would have been known to the skilled person as part of his common general

Samsung Bioepis Exhibit No. 1002
Page 7913

knowledge at the effective filing date (see, for example, D41, an extract from the 1994 textbook, Alberts *et al*). Therefore, the skilled person would have been able to make (the mature form of) eculizumab, and hence a single-use dosage form of a sterile and preservative free solution comprising 30ml of a 10mg eculizumab/ml solution, without an undue burden in 2007.

5 Auxiliary Requests

5.1 Auxiliary Request 1 specifies that administration is by intravenous infusion. Basis for this amendment can be found on page 22 of the application as filed. For instance, page 22 lines 18 to 20 indicate that intravenous administration is the most preferable route of administration, and page 22, line 32 also specifies intravenous infusion.

5.2 Auxiliary Request 2 specifies that administration is via IV infusion and that the composition is diluted to a final concentration of 5 mg/ml prior to administration. Basis for this amendment can be found at page 22, lines 27 to 29 of the application as filed.

5.3 Each of these Auxiliary Requests is filed in order to address the arguments raised by the Opponents in relation to inventive step.

6 Summary

6.1 For the reasons discussed above and in our previous response, the Proprietor requests that the patent is maintained as granted and the oppositions are dismissed.

Signed

AMANDA SIMONS
J A KEMP
ASSOCIATION NO. 811

PAMELA TUXWORTH
J A KEMP
ASSOCIATION NO. 811

Samsung Bioepis Exhibit No. 1002
Page 7914

AUXILIARY REQUEST 1

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein administration is by intravenous infusion.

Samsung Bioepis Exhibit No. 1002
Page 7915

AUXILIARY REQUEST 2

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein the composition is administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration.

Samsung Bioepis Exhibit No. 1002
Page 7916

AUXILIARY REQUEST 1

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein administration is by intravenous infusion.

Samsung Bioepis Exhibit No. 1002
Page 7917

AUXILIARY REQUEST 2

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH) wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein the composition is administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration.

Samsung Bioepis Exhibit No. 1002
Page 7918

MOLECULAR BIOLOGY OF
THE CELL
THIRD EDITION

Bruce Alberts • Dennis Bray
Julian Lewis • Martin Raff • Keith Roberts
James D. Watson



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Samsung Bioepis Exhibit No. 1002
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Front cover: The photograph shows a rat nerve cell in culture. It is labeled (*yellow*) with a fluorescent antibody that stains its cell body and dendritic processes. Nerve terminals (*green*) from other neurons (not visible), which have made synapses on the cell, are labeled with a different antibody. (Courtesy of Olaf Mundigl and Pietro de Camilli.)

Dedication page: Gavin Borden, late president of Garland Publishing, weathered in during his mid-1980s climb near Mount McKinley with MBoC author Bruce Alberts and famous mountaineer guide Mugs Stump (1940–1992).

Back cover: The authors, in alphabetical order, crossing Abbey Road in London on their way to lunch. Much of this third edition was written in a house just around the corner. (Photograph by Richard Olivier.)

tein translocator. There is now strong evidence for a protein translocator. Normally, the pore in the translocator is plugged with the growing polypeptide chain that is in transit across the membrane; when the nascent chains are experimentally released from the ribosomes with the drug puromycin, however, the pores can be detected by the ion currents that flow through them. Although the pore is large enough to allow the passage of an unfolded polypeptide chain, it closes when the ribosome is removed from the membrane (Figure 12–42). Thus the pore seems to be a dynamic structure, opening when a ribosome with a growing polypeptide chain attaches to the membrane and closing when the ribosome detaches after the synthesis of the protein is completed.

The ER Signal Peptide Is Removed from Most Soluble Proteins After Translocation [31]

We have seen that in chloroplasts and mitochondria the signal peptides are cleaved from the precursor proteins once they have crossed the membrane. Similarly, amino-terminal ER signal peptides are removed by a signal peptidase on the luminal side of the ER membrane. The peptide by itself, however, is not sufficient to signal cleavage by the peptidase; this requires an adjacent cleavage site that is specifically recognized by the peptidase. We shall see below that ER signal peptides that are contained within the polypeptide chain rather than at the amino terminus do not have these recognition sites and are never cleaved; instead, they can serve to retain transmembrane proteins in the lipid bilayer after the translocation process has been completed.

The amino-terminal ER signal peptide of a soluble protein itself has two signaling functions: in addition to directing the protein to the ER membrane, it is thought to serve as a **start-transfer signal,** which remains bound to the translocation apparatus while the rest of the protein is threaded continuously through the membrane as a large loop. Once the carboxyl terminus of the protein has passed through the membrane, the signal peptide is released from the translocator pore, cleaved off by the signal peptidase, and rapidly degraded to amino acids by other proteases in the ER while the protein is released into the ER lumen (Figure 12–43).

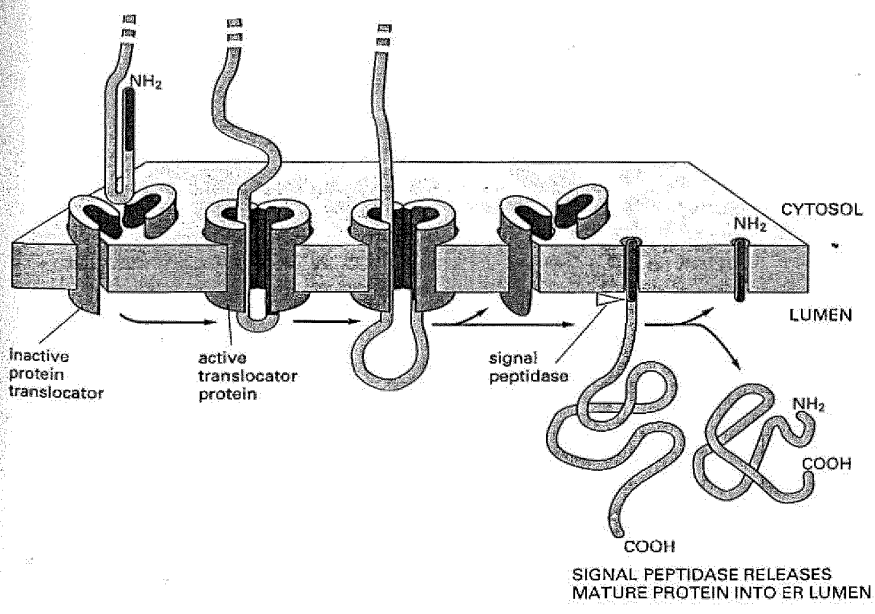


Figure 12–43 **The translocation of a soluble protein across the ER membrane.** In this hypothetical model the protein translocator in the membrane is postulated to exist in two alternative states—active or inactive. On binding an ER signal peptide (which acts as a start-transfer signal), the translocator adopts an active state and begins to transfer the polypeptide chain across the lipid bilayer as a loop. In this state it forms an aqueous pore across the membrane that can be detected electrophysiologically if the polypeptide chain is released (see Figure 12–42). After the protein has been completely translocated, the translocator reverts to an inactive conformation, which can no longer conduct ions across the membrane but is open to the lipid bilayer, allowing the hydrophobic signal peptide to diffuse out into the bilayer, where it is rapidly degraded. In this and the following two figures the ribosomes have been omitted for clarity.

Samsung Bioepis Exhibit No. 1002
Page 7921

IN THE MATTER OF
European Patent No. EP 2 359 834

DECLARATION OF PROFESSOR ALEXANDER M. KLIBANOV

I, Dr. Alexander M. Klibanov, hereby declare as follows:

1. I am a Professor of Chemistry and Bioengineering at the Massachusetts Institute of Technology ("M.I.T."), where I have been teaching and conducting research for more than 39 years. I currently hold the Novartis Endowed Chair Professorship. I obtained an M.S. degree in Chemistry in 1971 and a Ph.D. in Chemical Enzymology in 1974, both from Moscow University in Russia.

2. Throughout my career as a practicing chemist, I have extensively researched, published, taught, and lectured in many areas of biological, medicinal, protein, and pharmaceutical formulation chemistry. For my professional accomplishments, I have received numerous honors. For example, I was elected to the U.S. National Academy of Sciences and the U.S. National Academy of Engineering; I have also received various awards and prizes from the American Chemical Society, as well as other national and international organizations. I serve on the Editorial Boards of over a dozen scientific journals, including the "Journal of Biological Chemistry and Molecular Pharmacology" and the "International Journal of Drug Design, Delivery and Safety." I have published some 315 scientific papers in various chemical disciplines, including protein stability and stabilization, as well as drug formulation and delivery. I have also founded six pharmaceutical companies and have been on the scientific advisory boards and/or boards of directors of those companies, as well as of many others. A number of these advisory and directorship activities have involved the formulation, stability, stabilization, administration, and pharmacological evaluation of protein-based therapeutic agents, including antibodies. A copy of my curriculum vitae is attached as Exhibit 1.

3. I have been asked by Alexion to provide information regarding the field of protein, including antibody, pharmaceutical formulations as of 2007 and what understanding and expectations a person skilled in that field would have had concerning their stability at that time.

4. One of the major challenges in developing a successful pharmaceutical formulation of a therapeutic antibody (and of any therapeutic protein) around 2007 was, and still is today, being able to create a composition that is stable enough for sufficiently long periods of time such that the antibody does not undergo deleterious physical or chemical degradation reactions or form aggregates prior to administration. If the antibody is not adequately stable in the composition, serious problems can arise, such as drastically diminished efficacy and adverse immune responses in the patient. Moreover, when developing an antibody for commercial therapeutic use, it is often critical to be able to formulate the antibody at a rather high concentration (thus allowing a low-volume dosage).

5. Based on my professional experience, in the 2007 time-frame (or even today) there was no assurance that a skilled person, when provided with a new therapeutic protein, including an antibody, would have been able to develop a suitably stable pharmaceutical composition to make it commercially viable. My experience in this regard is confirmed by relevant scientific publications around that time period, as discussed below.

6. For example, a 2002 book entitled "Rational Design of Stable Protein Formulations. Theory and Practice" (the "2002 book;" Ex. 2) describes the uncertainty that existed in the protein formulation field regarding the ability to develop a suitably stable liquid pharmaceutical composition for a new protein. The authors of the 2002 book state that "protein drugs may not be stable enough to be handled as a liquid formulation" and concede that "with many proteins, it is not possible – especially considering the time constraints with product development – to develop sufficiently stable aqueous formulations." (Ex. 2 at 10 and 109.) The authors suggested that there was no reasonable expectation at the time of being able to develop such a composition, especially a liquid one, *e.g.*, stating that "[i]t can be *assumed* that most proteins will not exhibit sufficient stability in aqueous solution to allow a liquid formulation to be developed." (Ex. 2 at 188.) Consequently, most pharmaceutical proteins, such as antibodies, commercially available even today are in a lyophilized form, which requires their reconstitution prior to administration, thereby making it far less convenient and more expensive than aqueous solutions.

7. As the 2002 book recognized and was understood both then and now, numerous factors influence the stability of an antibody (or any protein), thus making the development of a suitable pharmaceutical composition in each new specific case a unique challenge. The book explains that "the exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation" and that "[e]ven for closely related proteins, the relative stability and major pathways for degradation might be quite different." (Ex. 2 at 185-186.) In the 2007 time-frame, researchers like myself understood that therapeutic antibody (or protein in general) formulations, especially liquid ones, are ***not*** amenable to a "one size fits all" approach. Therefore, when given a new antibody and tasked with creating its suitable pharmaceutical composition, a person skilled in the art would have had little guidance in that endeavor. Indeed, the 2002 book recognized that developing such a stable composition "is not a simple task" and that "for most proteins, maintaining physical and chemical stabilities in aqueous solution for an extended period of time is extremely difficult" (Ex. 2 at 10-11 and 184).

8. A review article entitled "Formulation and delivery issues for monoclonal antibody therapeutics" published in 2006 in a peer-reviewed international journal had recognized the uniqueness of each therapeutic antibody's challenge when developing a suitably robust formulation: "A priori, one might assume that by finding a stable formulation for one of these antibody drugs, that such a formulation would be good for most[,] if not all, similar antibodies. If this were borne out by experience, there would be no need for a review such as this. Instead, each antibody seems to have a unique personality related to its requirements for

stability." (D21 at 690). For this reason, proteins typically have to be evaluated individually and stabilized on an empirical basis, which inevitably leads to unpredictability.

9. Another, 2007 review paper entitled "Antibody structure, instability, and formulation" points out that "antibodies, like other proteins, may generate a variety of degradants during production, processing, and storage both in liquid and solid states. Their tendency to generate such degradants depends very much on individual sequence, pI, hydrophobicity, and carbohydrate content." (D20 at 14). The review noted numerous variables affecting each of the many causes of antibody instability; because they were, in turn, influenced by a number of intertwining factors, as well as by the structure of the protein molecule itself, a person skilled in the art in 2007 would have recognized that predicting in advance whether or how a new antibody could be stabilized under relevant conditions at a particular concentration in a pharmaceutical composition was not possible: "It should be stressed that one formulation excipient stabilizing a specific antibody may not be suitable for another because of the difference in their sequence." (D20 at 14).

10. Given the complexity and the sheer number of the various modes of protein instability and the diverse mechanisms underlying each of them, there was no universal approach to successfully formulating an antibody in 2007 (nor is there one now). My both academic and industrial experience at the time was that the field of antibody formulation was difficult, case-specific, and uncertain. For these reasons, a person skilled in the art aiming to develop a liquid formulation of a new antibody with a sufficient storage stability at a high concentration would ***not*** have been able to predict which, if any, such formulation would meet the necessary requirements.

I do hereby declare that the facts stated above are true to the best of my knowledge.



Dr. Alexander M. Klibanov

November 16, 2018

Date

Samsung Bioepis Exhibit No. 1002
Page 7924

Exhibit 1: Dr Klibanov Curriculum Vitae

Exhibit 2: Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8 (D40).

Samsung Bioepis Exhibit No. 1002
Page 7925

Curriculum vitae

ALEXANDER M. KLIBANOV

Date and Place of Birth: July 15, 1949, in Moscow (Russia)

Nationality: Naturalized U.S. Citizen (1983)

Education:

1974	Ph.D. in Chemical Enzymology, Moscow University
1971	M.S. in Chemistry, Moscow University

Honors:

2015	Tau Beta Pi's Leonardo Da Vinci Lecturer, MIT
2007-11 and 2014-	Novartis Chair Endowed Professorship, MIT
2012-13	Roger and Georges Firmenich Endowed Professorship, MIT
2011	MIT Biological Engineering Senior Class Faculty Award
2006	Distinguished GRUM Lecturer in Drug Discovery & Development, University of Montreal (Canada)
2004	UNAM Distinguished Lecturer, National University of Mexico (Mexico City)
2001	Walter Enz Lecturer in Pharmaceutical Chemistry, University of Kansas
2001	Elected a Corresponding Fellow of the Royal Society of Edinburgh (Scotland's National Academy of Science and Letters)
2000	Merck Distinguished Lecturer, Rutgers University
2000	Top 20 *Biotechnology & Bioengineering* Papers of the Last Forty Years
1998	Robert Lutz Lecturer, University of Virginia
1996	Perkin-Elmer Distinguished Lecturer, University of Pittsburgh
1995	Elected to the National Academy of Sciences of the U.S.A.
1995	Nathan O. Kaplan Memorial Lecturer in Biological Chemistry, University of California at San Diego
1994	R.W. Johnson PRI Lecturer, Pharmaceutical Research Institute
1994	Warren McCabe Lecturer, North Carolina State University
1993	Elected to the National Academy of Engineering of the U.S.A.
1993	Arthur C. Cope Scholar Award of the American Chemical Society
1993	Biotechnology Divisional Lectureship Award of the Institute of Food Technologists
1992	Charles Sabat Lecturer, Rutgers University
1992	Elected a Founding Fellow of the American Institute for Medical and Biological Engineering
1992	Louis C. Jordy Research Scholar Lecturer, Drew University
1991	International Enzyme Engineering Award
1991	Marvin J. Johnson Award of the American Chemical Society
1990	Monsanto Lecturer, Ohio State University
1990	NRC Distinguished Lecturer, Academia Sinica (Taiwan)
1989	Ipatieff Prize of the American Chemical Society
1989	Backer Lecturer, Groningen University (Holland)
1988	Dow Lecturer, University of Ottawa (Canada)
1987	Distinguished Scholar Lecturer, Hope College
1986	Leo Friend Award of the American Chemical Society
1984	Who's Who in Frontier Science and Technology

Momenta Pharm. v. BMS, IPR215-01537
1/41 BMS EXHIBIT 2016

2480

Samsung Bioepis Exhibit No. 1002
Page 7926

1984	Sohio Lecturer, Case Western Reserve University
1982	American Men and Women of Science
1981-1983	Henry L. Doherty Career Development Professorship, MIT
1975	U.S.S.R. Ministry of Higher Education Prize

Professional Experience:

2007-11 and 2014-	Novartis Chair Endowed Professor of Chemistry and Bioengineering, MIT
2012-2013	Roger and Georges Firmenich Professor of Natural Products Chemistry Department of Chemistry, MIT
2000-present	Professor of Bioengineering Department of Biological Engineering, MIT
1988-present	Professor of Chemistry Department of Chemistry, MIT
1987-1988	Professor of Applied Biochemistry Department of Applied Biological Sciences, MIT
1983-1987	Associate Professor of Applied Biochemistry Department of Applied Biological Sciences (formerly Department of Nutrition and Food Science), MIT
1979-1983	Assistant Professor of Applied Biochemistry Department of Nutrition and Food Science, MIT
1977-1979	Postdoctoral Associate, Department of Chemistry University of California at San Diego
1974-1977	Research Chemist Department of Chemistry, Moscow University

Current Journal Editorial/Advisory Boards:

"Biocatalysis and Biotransformation", "Applied Biochemistry and Biotechnology", "Central European Journal of Chemistry", "Biotechnology Progress", "Biotechnology & Bioengineering", "Microbial Biotechnology", "Open Journal of Pharmacology", "Nanocarriers", "Journal of Antivirals and Antiretrovirals", "Open Access Academic Books in Chemistry", "Journal of Biological Chemistry and Molecular Pharmacology", "Recent Patents in Biotechnology", "Current Pharmaceutical Biotechnology"

Professional Societies:

U.S. National Academy of Sciences, U.S. National Academy of Engineering, American Chemical Society, Society for Applied Microbiology

Current Research Interests:

Enzyme chemistry and biotechnology
Medicinal and formulation chemistry
Protein drug delivery
Antimicrobial polymers
Enzymes as stereoselective catalysts in organic syntheses
Stabilization and formulation of macromolecular pharmaceuticals

Momenta Pharm. v. BMS, IPR215-01537
2/41 BMS EXHIBIT 2016

2481

Samsung Bioepis Exhibit No. 1002
Page 7927

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2. Varfolomeyev, S.D., Klibanov, A.M., Martinek, K., Berezin, I.V. 1972. Light-sensitive catalysts. 4-Nitrocinnamoyl residue as a chromophoric reporter group in the α-chymotrypsin active center. Dokl. Acad. Nauk SSSR 203: 616-619.

3. Klibanov, A.M., Samokhin, G.P., Martinek, K., Berezin, I.V. 1974. Mechanochemistry of catalytic systems. Regulation by a mechanical action of the enzymatic properties of α-chymotrypsin covalently attached to a nylon fiber. Dokl. Acad. Nauk SSSR 218: 715-718.

4. Berezin, I.V., Varfolomeyev, S.D., Klibanov, A.M., Martinek, K. 1974. Light and ultrasonic regulation of α-chymotrypsin catalytic activity. Proflavin as a light- and sound- sensitive competitive inhibitor. FEBS Lett. 39: 329-331.

5. Berezin, I.V., Klibanov, A.M., Martinek, K. 1974. The mechanochemistry of immobilized enzymes. How to steer a chemical process at the molecular level by a mechanical device. Biochim. Biophys. Acta 364: 193-199.

6. Berezin, I.V., Klibanov, A.M., Goldmacher, V.S., Martinek, K. 1974. Mechanochemistry of catalytic systems. Regulation by a mechanical action of the enzymatic activity of trypsin entrapped in polyacrylamide gel. Dokl. Acad. Nauk SSSR 218: 367-370.

7. Klibanov, A.M., Martinek, K., Berezin, I.V. 1974. The effect of ultrasound on α-chymotrypsin. A novel approach to studying conformational transitions in active centers of enzymes. Biochemistry SSSR 39: 878-887.

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10. Martinek, K., Goldmacher, V.S., Klibanov, A.M., Berezin, I.V. 1975. Denaturing agents (urea, acrylamide) protect enzymes against irreversible thermoinactivation: a study with native and immobilized α-chymotrypsin and trypsin. FEBS Lett. 51: 152-155.

11. Martinek, K., Klibanov, A.M., Tchernysheva, A.V., Berezin, I.V. 1975. The stabilization of α-chymotrypsin by entrapment in polymethacrylate gels. Dokl. Acad. Nauk SSSR 223: 233-236.

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Momenta Pharm. v. BMS, IPR215-01537
3/41 BMS EXHIBIT 2016

2482

Samsung Bioepis Exhibit No. 1002
Page 7928

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Momenta Pharm. v. BMS, IPR215-01537
4/41 BMS EXHIBIT 2016

2483

Samsung Bioepis Exhibit No. 1002
Page 7929

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Momenta Pharm. v. BMS, IPR215-01537
5/41 BMS EXHIBIT 2016

2484

Samsung Bioepis Exhibit No. 1002
Page 7930

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Momenta Pharm. v. BMS, IPR215-01537
6/41 BMS EXHIBIT 2016

2485

Samsung Bioepis Exhibit No. 1002
Page 7931

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Momenta Pharm. v. BMS, IPR215-01537
7/41 BMS EXHIBIT 2016

2486

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Page 7932

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Momenta Pharm. v. BMS, IPR215-01537
8/41 BMS EXHIBIT 2016

2487

Samsung Bioepis Exhibit No. 1002
Page 7933

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Momenta Pharm. v. BMS, IPR215-01537
9/41 BMS EXHIBIT 2016

2488

Samsung Bioepis Exhibit No. 1002
Page 7934

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2489

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2490

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12/41 BMS EXHIBIT 2016

2491 AMG1027 (pt. 7)

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13/41 BMS EXHIBIT 2016

2492

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2493

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2494

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2495

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2496

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2497

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19/41 BMS EXHIBIT 2016

2498

Samsung Bioepis Exhibit No. 1002
Page 7944

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2499

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23/41 BMS EXHIBIT 2016

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24/41 BMS EXHIBIT 2016

2503

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Page 7949

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Invited Lectures:

1. Gordon Conference on Immobilized Enzymes, Complexes, and Cells (Plymouth, NH). August 1978.

2. 5th International Conference on Enzyme Engineering (Henniker, NH). August 1978.

Momenta Pharm. v. BMS, IPR215-01537
25/41 BMS EXHIBIT 2016

2504

Samsung Bioepis Exhibit No. 1002
Page 7950

3. Miles Laboratories (Elkhart, IN). February 1980.
4. Gordon Conference on Immobilized Species (Henniker, NH). August 1980.
5. Novo Laboratories (Wilton, CT). October 1980.
6. Corning Glass Works Co. (Corning, NY). October 1980.
7. Stauffer Chemical Co. (Dobbs Ferry, NY). December 1980.
8. DuPont Experimental Station (Wilmington, DE). January 1981.
9. Dow Chemical Co. (Wayland, MA). April 1981.
10. Cetus Corp. (Berkeley, CA). April 1981.
11. Exxon Research & Engineering Co. (Linden, NJ). April 1981.
12. Meeting of the American Solar Energy Society (Philadelphia, PA). May 1981.
13. 6th International Conference on Enzyme Engineering (Kashikojima, Japan). September 1981.
14. Genex Corp. (Rockville, MD). October 1981.
15. ILP Symposium for Senior Executives (London, England). November 1981.
16. California Institute of Technology (Pasadena, CA). December 1981.
17. Symposium of the American Society for Microbiology (Atlanta, GA). March 1982.
18. Kodak Research Center (Rochester, NY). March 1982.
19. Repligen Corp. (Cambridge, MA). April 1982.
20. Symposium on the Biological Basis of New Developments in Biotechnology (Minneapolis, MN). May 1982.
21. Meeting of the Biochemical Society (Oxford, England). July 1982.
22. Meeting of the American Chemical Society (Kansas City, MO). September 1982.
23. Rotenburger Symposium on Enzyme Technology (Kassel, Germany). September 1982.
24. Genetics Institute (Boston, MA). September 1982.
25. Meeting of the American Institute of Chemical Engineers (Los Angeles, CA). November 1982.
26. G.D. Searle Co. (Chicago, IL). October 1982.
27. Halcon International Research Center (Montvale, NJ). October 1982.

Momenta Pharm. v. BMS, IPR215-01537
26/41 BMS EXHIBIT 2016

2505

Samsung Bioepis Exhibit No. 1002
Page 7951

28. International Workshop on Immobilized Enzymes and Cells (Bangkok, Thailand). December 1982.

29. Amoco Research Center (Naperville, IL). January 1983.

30. University College London (London, England). May 1983.

31. International Conference on Biotechnology Biotech '83 (London, England). May 1983.

32. Tufts University Workshop on Immobilized Enzymes and Proteins (Medford, MA). June 1983.

33. Gordon Conference on Organic Reactions and Processes (New Hampton, NH). July 1983.

34. Symposium "Biocatalysis: New Discoveries and Uses", Meeting of the American Society for Industrial Microbiology (Sarasota, FL). August 1983.

35. Monsanto Co. (St. Louis, MO). September 1983.

36. Pasteur Biosciences '83 Conference (Paris, France). September 1983.

37. 7th International Conference on Enzyme Engineering (White Haven, PA). September 1983.

38. University of Iowa (Iowa City, IA). October 1983.

39. CPC Moffett Technical Center (Summitt-Argo, IL). December 1983.

40. General Foods Co. (Dobbs Ferry, NY). December 1983.

41. U.S. National Bureau of Standards (Gaithersburg, MD). April 1984.

42. Millipore Corp. (Bedford, MA). April 1984.

43. Technical University of Munich (Munich, F.R.G.). May 1984.

44. Danish Academy of Technical Sciences (Copenhagen, Denmark). May 1984.

45. Novo International Biotechnology Symposium (Copenhagen, Denmark). May 1984.

46. CRA Scientific Conference (St. Charles, IL). June 1984.

47. Gordon Conference on Immobilized Species in Biotechnology (Plymouth, NH). August 1984.

48. Meeting of the American Chemical Society (Philadelphia, PA). August 1984.

49. International Biotechnology Conference Biotech '84 (Washington, DC). September 1984.

50. Staley Manufacturing Co. (Decatur, IL). October 1984.

51. Case Western Reserve University (Cleveland, OH). October 1984.

Momenta Pharm. v. BMS, IPR215-01537
27/41 BMS EXHIBIT 2016

2506

Samsung Bioepis Exhibit No. 1002
Page 7952

52. IMC Corp. (Terre Haute, IN). October 1984.

53. Syracuse University (Syracuse, NY). November 1984.

54. National University of Mexico (Mexico City, Mexico). November 1984.

55. National Starch & Chemical Corp. (Bridgewater, NJ). November 1984.

56. Mead Corp. (Chillicothe, OH). November 1984.

57. Hebrew University of Jerusalem (Jerusalem, Israel). November 1984.

58. U.S. Army Chemical R&D Center (Aberdeen Proving Ground, MD). December 1984.

59. Allied Corp. (Morristown, NJ). December 1984.

60. Bristol Laboratories (Syracuse, NY). January 1985.

61. University of Rochester (Rochester, NY). January 1985.

62. G.D. Searle Co. (Chicago, IL). February 1985.

63. Wellcome Research Laboratories (Research Triangle Park, NC). February 1985.

64. UCLA Symposium on Protein Structure, Folding and Design (Keystone, CO). April 1985.

65. International Conference on Application of Biocatalysts in Organic Synthesis (Noordwijkerhout, Holland). April 1985.

66. Guido Donegani Research Institute (Novara, Italy). April 1985.

67. Hoffmann-LaRoche Co. (Basel, Switzerland). April 1985.

68. Pittsburgh-Cleveland Catalysis Society Meeting (Pittsburgh, PA). May 1985.

69. Ethyl Corp. (Baton Rouge, LA). May 1985.

70. Stony Brook Symposium on Protein Engineering (Stony Brook, NY). May 1985.

71. Amgen Corp. (Thousand Oaks, CA). June 1985.

72. Gordon Conference on Applied Microbiology (New London, NH). August 1985.

73. International Congress of Biochemistry (Amsterdam, Holland). August 1985.

74. IUPAC Congress (Manchester, England). September 1985.

75. International Enzyme Engineering Conference (Helsingor, Denmark). September 1985.

76. Biotechnica '85 (Hannover, West Germany). October 1985.

Momenta Pharm. v. BMS, IPR215-01537
28/41 BMS EXHIBIT 2016

2507

Samsung Bioepis Exhibit No. 1002
Page 7953

77. Biotec 85 (Dusseldorf, West Germany). October 1985.

78. Smith Kline & French Co. (Philadelphia, PA). November 1985.

79. Exxon Corporate Research (Anandale, NJ). December 1985.

80. Pennsylvania State University (University Park, PA). December 1985.

81. Georgetown University (Washington, DC). February 1986.

82. Columbia University (New York, NY). February 1986.

83. Upjohn Co. (Kalamazoo, MI). February 1986.

84. UNIDO Workshop on Biotechnology (Trieste, Italy). March 1986.

85. Enericherche (Monterotondo, Italy). March 1986.

86. EEC Biomolecular Engineering Meeting (Compiegne, France). April 1986.

87. ACS Conference on Chemical Aspects of Biotechnology (Gaithersburg, MD). May 1986.

88. Biotech '86 Europe International Conference (London, England). May 1986.

89. IUPAC International Symposium on Organic Chemistry in Technological Perspective (Jerusalem, Israel). June 1986.

90. University of Tel-Aviv (Ramat-Aviv, Israel). June 1986.

91. Annual Meeting of the Canadian Biochemical Society (Guelph, Canada). June 1986.

92. International Symposium on Biologically Engineered Polymers (Cambridge, England). July 1986.

93. TNO Institute of Applied Chemistry (Zeist, The Netherlands). August 1986.

94. IUPAC International Symposium on the Chemistry of Natural Products (The Hague, The Netherlands). August 1986.

95. American Chemical Society National Meeting (Anaheim, CA). September 1986.

96. Catalytica Annual Science and Technology Symposium (San Jose, CA). September 1986.

97. International Conference on Enzyme Engineering (Cambridge, England). September 1986.

98. Ohio State University (Columbus, OH). October 1986.

99. Michigan State University (Lansing, MI). October 1986.

100. Biotechnology Process Engineering Symposium (Cambridge, MA). October 1986.

Momenta Pharm. v. BMS, IPR215-01537
29/41 BMS EXHIBIT 2016

2508

Samsung Bioepis Exhibit No. 1002
Page 7954

101. University of Delaware (Newark, DE). November 1986.

102. American Red Cross Blood Research Institute (Bethesda, MD). November 1986.

103. International Symposium on Biocatalysis in Non-Aqueous Solvents (Wageningen, The Netherlands). December 1986.

104. University of Trondheim (Trondheim, Norway). December 1986.

105. Colgate-Palmolive Research Center (Piscataway, NJ). January 1987.

106. International Paper Co. (Tuxedo Park, NY). January 1987.

107. University of Massachusetts (Amherst, MA). February 1987.

108. American Society for Microbiology Biocatalysis Symposium (Atlanta, GA). March 1987.

109. Hope College (Holland, MI). March 1987.

110. Pfizer Corporate Research Center (Groton, CT). April 1987.

111. OBBS Symposium on Protein Engineering (Ottawa, Canada). April 1987.

112. University of Texas (Austin, TX). April 1987.

113. Florida Catalysis Conference (Palm Coast, FL). May 1987.

114. PPG Industries (Barberton, OH). May 1987.

115. Abbott Laboratories (North Chicago, IL). May 1987.

116. American Oil Chemists Society Symposium on Lipases (New Orleans, LA). May 1987.

117. Argonne National Laboratory (Argonne, IL). June 1987.

118. Canadian Chemical Conference (Quebec City, Canada). June 1987.

119. Squibb Co. (New Brunswick, NJ). June 1987.

120. FEBS Annual Meeting (Ljubljana, Yugoslavia). July 1987.

121. L'Oreal Research Institute (Paris, France). July 1987.

122. International Symposium on Biointeractions (Cambridge, England). July 1987.

123. Eli Lilly Co. (Indianapolis, IN). August 1987.

124. Upjohn Co. (Kalamazoo, MI). September 1987.

125. International Enzyme Engineering Conference (Santa Barbara, CA). October 1987.

Momenta Pharm. v. BMS, IPR215-01537
30/41 BMS EXHIBIT 2016

2509

Samsung Bioepis Exhibit No. 1002
Page 7955

126. Shell Development Co. (Houston, TX). October 1987.

127. DuPont Experimental Station (Wilmington, DE). October 1987.

128. University of Helsinki (Helsinki, Finland). November 1987.

129. International Conference on Bioreactors and Biotransformations (Gleneagles, Scotland). November 1987.

130. Sandoz Co. (Basel, Switzerland). November 1987.

131. University of Waterloo (Waterloo, Canada). December 1987.

132. Norwegian Biochemical Society Annual Meeting (Bejito, Norway). January 1988.

133. Rutgers University (New Brunswick, NJ). February 1988.

134. University of Ottawa (Ottawa, Canada). February 1988.

135. Boston Biomedical Research Institute (Boston, MA). March 1988.

136. Princeton University (Princeton, NJ). March 1988.

137. Royal Society of Chemistry Meeting (Kent, England). April 1988.

138. Stanford University (Stanford, CA). May 1988.

139. University of Graz (Graz, Austria). May 1988.

140. Belgian Organic Chemistry Symposium (Ghent, Belgium). May 1988.

141. Dow Chemical Co. (Midland, MI). May 1988.

142. Michigan State University (Lansing, MI). May 1988.

143. American Chemical Society Meeting (Toronto, Canada). June 1988.

144. Proctor & Gamble Co. (Cincinnati, OH). June 1988.

145. International Congress of Biochemistry (Prague, Czechoslovakia). July 1988.

146. International Biotechnology Symposium (Paris, France). July 1988.

147. Gordon Conference on Catalysis (Newport, RI). August 1988.

148. Conference on Biocatalytic Synthesis of Organic Compounds (Saratoga, NY). August 1988.

149. International Symposium on Thermodynamics Applied to Biological Systems (Santa Margherita, Italy). September 1988.

150. Cornell University (Ithaca, NY). September 1988.

Momenta Pharm. v. BMS, IPR215-01537
31/41 BMS EXHIBIT 2016

2510

Samsung Bioepis Exhibit No. 1002
Page 7956

151. Eastman Kodak Co. (Kingsman, TN). September 1988.

152. Symposium on Biotechnology Challenges in the Food Industry (Williamsburg, VA). October 1988.

153. University of California at Irvine (Irvine, CA). October 1988.

154. UCSF Symposium on Protein and Drug Design (San Francisco, CA). October 1988.

155. Boston University (Boston, MA). October 1988.

156. Wayne State University (Detroit, MI). November 1988.

157. Kyoto Symposium on Frontiers in Biocatalysis (Kyoto, Japan). November 1988.

158. Rohm & Haas Co. (Spring House, PA). January 1989.

159. Block Drug Co. (Jersey City, NJ). January 1989.

160. State University of New York (Albany, NY). March 1989.

161. Scripps Clinic and Research Foundation (La Jolla, CA). April 1989.

162. Texas A&M University (College Station, TX). May 1989.

163. University of Texas (Austin, TX). May 1989.

164. International Congress of Food Engineering (Cologne, F.R.G.). May 1989.

165. University of Groningen (Groningen, The Netherlands). June 1989.

166. Gordon Conference on Enzymes, Coenzymes, and Metabolic Pathways (Meridan, NH). July 1989.

167. American Peptide Symposium (La Jolla, CA). July 1989.

168. International Symposium on Prospects in Protein Engineering (Haren, The Netherlands). August 1989.

169. Connaught Laboratories (Toronto, Canada). September 1989.

170. University of Georgia (Athens, GA). October 1989.

171. Spanish National Congress of Biochemistry (Alicante, Spain). October 1989.

172. Symposium on Receptor- and Enzyme-Based Drug Design (Chapel Hill, NC). October 1989.

173. Wesleyan University (Middletown, CT). November 1989.

174. University of Wisconsin (Madison, WI). February 1990.

175. Merck Sharp & Dohme Research Laboratories (Rahway, NJ). February 1990.

Momenta Pharm. v. BMS, IPR215-01537
32/41 BMS EXHIBIT 2016

2511

Samsung Bioepis Exhibit No. 1002
Page 7957

176. International Symposium on Biochemical Engineering (Stuttgart, F.R.G.). March 1990.

177. University of Geneva (Geneva, Switzerland). March 1990.

178. University of Lausanne (Lausanne, Switzerland). March 1990.

179. University of Bern (Bern, Switzerland). March 1990.

180. University of Fribourg (Fribourg, Switzerland). March 1990.

181. International Conference on Protein Stability (Cambridge, England). March 1990.

182. AFRC Institute of Food Research (Reading, England). March 1990.

183. Lehigh University (Bethlehem, PA). April 1990.

184. Biotechnology Research Institute (Montreal, Canada). April 1990.

185. Ohio State University (Columbus, OH). April 1990.

186. Pharmaceutical Manufacturers Association Annual Meeting (St. Louis, MO). April 1990.

187. International Conference on Industrial Use of Enzymes (Chicago, IL). May 1990.

188. Korea Advanced Institute of Science and Technology (Seoul, Korea). June 1990.

189. Academia Sinica and National Taiwan University (Taipei, Taiwan). June 1990.

190. Gordon Conference on Biocatalysis (Plymouth, NH). June 1990.

191. IUPAC Conference on Organic Synthesis (Helsinki, Finland). July 1990.

192. International Conference on Solution Chemistry (Ottawa, Canada). August 1990.

193. Gordon Conference on Biomolecular Recognition (Plymouth, NH). August 1990.

194. University of Naples (Naples, Italy). September 1990.

195. International Conference on Water and Life (Crans-sur-Sierre, Switzerland). September 1990.

196. International Symposium on Thermodynamic Basis of Protein Structure and Function (Kansas City, MO). October 1990.

197. Johns Hopkins University (Baltimore, MD). October 1990.

198. Institute of Medicine Workshop on Microheterogeneity of Biological Macromolecules (Arlington, VA). November 1990.

199. U.S.-Japan Biotechnology Conference (Honolulu, HI). January 1991.

Momenta Pharm. v. BMS, IPR215-01537
33/41 BMS EXHIBIT 2016

2512

Samsung Bioepis Exhibit No. 1002
Page 7958

200. Ciba-Geigy Corp. (Summit, NJ). February 1991.

201. City University of New York (New York, NY). March 1991.

202. Rensselaer Polytechnic Institute (Troy, NY). March 1991.

203. Bristol-Myers Squibb Co. (Wallingford, CT). April 1991.

204. British Biochemical Society Meeting (Reading, England). April 1991.

205. University of California at Berkeley (Berkeley, CA). April 1991.

206. California Institute of Technology (Pasadena, CA). April 1991.

207. Merck, Sharp & Dohme Research Laboratories (West Point, PA). May 1991.

208. Bristol-Myers Squibb Research Institute (Princeton, NJ). May 1991.

209. International Conference on Biocatalysis (Orlando, FL). June 1991.

210. U.S. Army Research, Development and Engineering Center (Natick, MA). July 1991.

211. Gordon Conference on Natural Products (Plymouth, NH). July 1991.

212. Lederle-Praxis Biologicals Co. (Sanford, NC). July 1991.

213. International Biochemistry Congress (Jerusalem, Israel). August 1991.

214. American Chemical Society Meeting (New York, NY). August 1991.

215. International Enzyme Engineering Conference (Kona, HI). September 1991.

216. University of Lowell (Lowell, MA). October 1991.

217. Rutgers University (Piscataway, NJ). November 1991.

218. International Symposium on Enzymes in Organic Synthesis (New Delhi, India). January 1992.

219. International BioSymposium Nagoya '92 (Nagoya, Japan). January 1992.

220. International Symposium on Biomolecules in Organic Solvents (Taxco, Mexico). February 1992.

221. Rockefeller University (New York, NY). March 1992.

222. Symposium on Molecular Chirality of the Pharmaceutical Society of Japan (Tokyo, Japan). April 1992.

223. Conference on Enzymes in Organic Chemistry of the Dutch Chemical Society (Wageningen, The Netherlands). April 1992.

Momenta Pharm. v. BMS, IPR215-01537
34/41 BMS EXHIBIT 2016

2513

Samsung Bioepis Exhibit No. 1002
Page 7959

224. International Symposium on Bioprocessing of Coal (Clearwater Beach, FL). May 1992.

225. Exxon Workshop on New Leads in Coal Depolymerization (Montgomery, TX). May 1992.

226. Italian Symposium on Biochemical Biotechnology (Capri, Italy). June 1992.

227. Gordon Conference on Biocatalysis (Meriden, NH). July 1992.

228. FEBS Meeting (Dublin, Ireland). August 1992.

229. International Biotechnology Symposium (Washington, DC). August 1992.

230. International Symposium Bio Japan '92 (Yokohama, Japan). August 1992.

231. Texas College of Osteopathic Medicine (Fort Worth, TX). October 1992.

232. Rutgers University (Newark, NJ). October 1992.

233. Schering-Plough Co. (Bloomfield, NJ). November 1992.

234. International Symposium ISOPOW-V: *Properties of Water in Foods* (Peniscola, Spain). November 1992.

235. Carlsberg Laboratory (Copenhagen, Denmark). November 1992.

236. International Food Technology Conference (The Hague, The Netherlands). November 1992.

237. Singapore Institute of Standards and Industrial Research (Singapore). November 1992.

238. Drew University (Madison, NJ). December 1992.

239. Northeastern University (Boston, MA). January 1993.

240. Ciba Corning Diagnostics Corp. (Irvine, CA). January 1993.

241. University of Kansas (Lawrence, KS). January 1993.

242. 6th International Symposium on Recent Advances in Drug Delivery Systems (Salt Lake City, UT). February 1993.

243. Pennsylvania State University (University Park, PA). March 1993.

244. Northwestern University (Evanston, IL). March 1993.

245. International Symposium on Technologies for the Production of Enantiomerically Pure Chemicals (Amelia Island, FL). March 1993.

246. Gordon Conference on Bioanalytical Sensors (Ventura, CA). March 1993.

247. Pharmaceutical Manufacturers Association Analytical R&D/Pharmaceutical Development Subsections Joint Annual Meeting (Lake Buena Vista, FL). April 1993.

Momenta Pharm. v. BMS, IPR215-01537
35/41 BMS EXHIBIT 2016

2514

Samsung Bioepis Exhibit No. 1002
Page 7960

248. University of Massachusetts Symposium on Breakthrough Technologies: *Paradigms for a New Century* (Amherst, MA). May 1993.

249. Parke-Davis Corp. (Ann Arbor, MI). May 1993.

250. NutraSweet Co. (Mt. Prospect, IL). June 1993.

251. IGT/GRI Workshop on Novel Concepts for the Production of Regio- and Stereospecific Compounds (Chicago, IL). June 1993.

252. Northeast Regional Meeting of the American Chemical Society (Boston, MA). June 1993.

253. Institute of Food Technologists' Biotechnology Meeting (Chicago, IL). July 1993.

254. Annual Meeting of Department of Energy's Biological and Chemical Technology Research Program Principal Investigators (Tiburon, CA). July 1993.

255. Allied-Signal Corp. (Des Plains, IL). July 1993.

256. American Chemical Society Meeting (Chicago, IL). August 1993.

257. University of Illinois at Urbana-Champaign (Urbana, IL). September 1993.

258. International Enzyme Engineering Conference (Deauville, France). September 1993.

259. Queens College (Flushing, NY). November 1993.

260. Exxon Chemical Research Meeting (Galveston, TX). November 1993.

261. Hoffmann-LaRoche Co. (Nutley, NJ). December 1993.

262. U.S.-Japan Symposium on Drug Delivery (Maui, HI). December 1993.

263. North Dakota State University (Fargo, ND). February 1994.

264. Annual Meeting of the American Institute for Medical and Biological Engineering (Washington, DC). March 1994.

265. Rhone-Poulenc Rorer Symposium on Visions in Chemistry (Collegeville, PA). May 1994.

266. International Flavors & Fragrances R&D (Union Beach, NJ). May 1994.

267. Istituto degli Ormoni (Milan, Italy). June 1994.

268. Gordon Conference on Enzymes, Coenzymes, and Metabolic Pathways (Meriden, NH). July 1994.

269. Annual Meeting of Department of Energy's Biological and Chemical Technology Research Program Principal Investigators (Denver, CO). July 1994.

270. William S. Johnson Symposium in Organic Chemistry (Stanford, CA). October 1994.

Momenta Pharm. v. BMS, IPR215-01537
36/41 BMS EXHIBIT 2016

2515

Samsung Bioepis Exhibit No. 1002
Page 7961

271. R.W. Johnson Pharmaceutical Research Institute (Spring House, PA). October 1994.

272. International Congress of Biochemical Engineering (Mexico City, Mexico). October 1994.

273. National University of Mexico (Mexico City, Mexico). October 1994.

274. DuPont Merck Pharmaceutical Co. (Deepwater, NJ). December 1994.

275. Symposium on Therapeutic Protein Production and Processing (Cambridge, MA). January 1995.

276 International Symposium on Biocatalysts for Flavor Production, BioFlavour '95 (Dijon, France). February 1995.

277. Institut Pasteur (Paris, France). February 1995.

278. Florida State University (Tallahassee, FL). March 1995.

279. Abbott Laboratories (North Chicago, IL). March 1995.

280. University of Maryland, Baltimore County (Baltimore, MD). March 1995.

281 University of California at San Diego (La Jolla, CA). April 1995.

282. Great Lakes Regional Meeting of the American Chemical Society (LaCrosse, WI). June 1995.

283. Gordon Conference on Bioorganic Chemistry (Andover, NH). June 1995.

284. Annual Meeting of Department of Energy's Biochemical and Chemical Technology Research Principal Investigators (Albuquerque, NM). July 1995.

285. International Symposium "Biocatalysis-95" (Suzdal, Russia). August 1995.

286. International Enzyme Engineering Conference (San Diego, CA). October 1995.

287. Symposium on Biomacromolecules: *From 3-D Structure to Applications* (Pasco, WA). October 1995.

288. Enzymol International Inc. (Columbus, OH). December 1995.

289. International Symposium Pacifichem '95 (Honolulu, HI). December 1995.

290. 3rd U.S.-Japan Symposium on Drug Delivery (Maui, HI). December 1995.

291. New England Section of the Society for Industrial Microbiology (Cambridge, MA). February 1996.

292. Brown University (Providence, RI). February 1996.

293. University of Pittsburgh (Pittsburgh, PA). February 1996.

294. Food Research Institute (Reading, England). March 1996.

Momenta Pharm. v. BMS, IPR215-01537
37/41 BMS EXHIBIT 2016

2516

Samsung Bioepis Exhibit No. 1002
Page 7962

295. International Symposium Chiral USA '96 (Boston, MA). May 1996.

296. BP Catalyst Colloquium (Uxbridge, England). June 1996.

297. International Symposium on Homogeneous Catalysis (Princeton, NJ). August 1996.

298. Juan March Foundation's International Workshop on Novel Biocatalysts (Madrid, Spain). March 1997.

299. University of Kentucky (Lexington, KY). April 1997.

300. Gordon Conference on Organic Reactions and Processes (Henniker, NH). August 1997.

301. Novo Nordisk Symposium on Protein Stability (Klampenborg, Denmark). August 1997.

302. International Enzyme Engineering Conference (Beijing, China). October 1997.

303. Biogen Inc. (Cambridge, MA). December 1997.

304. INBIO Europe'98 Conference on Advances in Industrial Biocatalysis (Amsterdam, The Netherlands). February 1998.

305. International Symposium on Stability and Stabilization of Biocatalysts (Córdoba, Spain). April 1998.

306. International Symposium on Protein Structure, Stability and Folding: *Fundamental and Medical Aspects* (Moscow, Russia). June 1998.

307. International Conference on Protein Stabilization (Leeds, England). June 1998.

308. University of Virginia (Charlottesville, VA). September 1998.

309. Danish Biotechnology Conference (Vejle, Denmark). May 1999.

310. International Conference on Biocatalysis and Biotransformation Biotrans '99 (Giardini Naxos, Italy). September 1999.

311. Annual Meeting of the American Association of Pharmaceutical Scientists (New Orleans, LA). November 1999.

312. Brandeis University (Waltham, MA). November 1999.

313. Rutgers University (Piscataway, NJ). March 2000.

314. Chiral USA 2000 Conference (Boston, MA). May 2000.

315. Land O'Lakes Conference on Solid-State Reactivity in Pharmaceutical Systems (Merrimac, WI). June 2000.

316. Agouron Pharmaceuticals Inc. (San Diego, CA). July 2000.

Momenta Pharm. v. BMS, IPR215-01537
38/41 BMS EXHIBIT 2016

2517

Samsung Bioepis Exhibit No. 1002
Page 7963

317. U.S. Patent and Trademark Office Technology Fair (Arlington, VA). July 2000.

318. 23rd Gulf Coast Chemistry Conference (Pensacola, FL). September 2000.

319. Avon Products (Suffern, NY). November 2000.

320. Procter & Gamble Co. (Cincinnati, OH). November 2000.

321. Flamel Technologies (Venissieux, France). December 2000.

322. BioVision 2001 International Conference (Lyon, France). February 2001.

323. 9th Baxter Science and Technology Symposium (Hyland, IL). May 2001.

324. International Symposium on Applications of Enzymes in Chemical and Biological Defense (Orlando, FL). May 2001.

325. International Conference on Applied Biocatalysis (Trondheim, Norway). June 2001.

326. PPG Industries (Allison Park, PA). August 2001.

327 University of Kansas (Lawrence, KS). August 2001.

328. Alkermes Inc. (Cambridge, MA). October 2001.

329. 6th U.S.-Japan Symposium on Drug Delivery Systems (Maui, HI). December 2001.

330. Acambis Inc. (Cambridge, MA). January 2002.

331. Baxter BioScience Co. (Duarte, CA). January 2002.

332. International Symposium on Challenges in Drug Discovery and Development in the 21st Century (Kolkata, India). March 2002.

333. National Institute of Standards and Technology (Gaithersburg, MD). April 2002.

334. Albany Molecular Research, Inc. (Mt. Prospect, IL). April 2002.

335. Institute for International Research Conference on Protein and Peptide Formulation Strategies for Drug Development and Delivery (San Francisco, CA). August 2002.

336. R&D Management Council Meeting of the Technical Association of the Pulp and Paper Industry (Cambridge, MA). November 2002.

337. DuPont Central R&D Center (Wilmington, DE). December 2002.

338. *ACTIVE*Pack 2003 Conference (Ponte Verde Beach, FL). February 2003.

339. International Symposium on Industrial Applications of Biocatalysis: *Chemical Development Issues* (Boston, MA). September 2003.

Momenta Pharm. v. BMS, IPR215-01537
39/41 BMS EXHIBIT 2016

2518

Samsung Bioepis Exhibit No. 1002
Page 7964

340. U.S. Army Workshop on Self-Decontaminating Materials and Multifunctional Coatings (Arlington, VA). October 2003.

341. British Royal Society Meeting on Biocatalysis in Alternative Media (London, England). December 2003.

342. National Autonomous University of Mexico (Mexico City, Mexico). March 2004.

343. Healthcare Research Initiative Workshop on the Effect of Radiofrequency Radiation on Pharmaceutical Product Quality (Cambridge, MA). June 2004.

344. Rensselaer Biotechnology Symposium on Biological Discoveries That Will Change the World (Troy, NY). September 2004.

345. Barnett International Conference: *The Consequences of Aggregation in Drug Development, Immunology, Quality Control, and Disease Genesis* (Philadelphia, PA). September 2004.

346. 4th Annual IBC Conference on Formulation Strategies for Protein Therapeutics (Boston, MA). October 2004.

347. U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD). August 2005.

348. World Congress on Synthetic Receptors (Salzburg, Austria). September 2005.

349. International Conference "Celebrating 30 Years of Robert Langer's Science" (Cambridge, MA). July 2006.

350. University of Montreal (Montréal, Québec, Canada). October 2006.

351. The Boeing Company Research Center (Everett, WA). December 2006.

352. Instrumentation Laboratory (Lexington, MA). December 2006.

353. Acambis Inc. (Cambridge, MA). January 2007.

354. 6th International Symposium on Industrial Microbiology and Biotechnology (Cambridge, MA). August 2007.

355. Scientific Conference on Chemical & Biological Defense Research (Timonium, MD). November 2007.

356. MIT Research and Development Conference (Cambridge, MA). November 2007.

357. Nanotech '2008 International Conference (Boston, MA). June 2008.

358. Materials Research Society Symposium (Boston, MA). December 2008.

359. Johnson & Johnson Research Briefing at MIT (Cambridge, MA). January 2009.

360. 3rd Annual Lyophilization Conference: *Ensuring Optimum Formulations for Pharmaceuticals*

Momenta Pharm. v. BMS, IPR215-01537
40/41 BMS EXHIBIT 2016

2519

Samsung Bioepis Exhibit No. 1002
Page 7965

and Biologics (Boston, MA). February 2010.

361. DSM Corporate Research Center (Geleen, The Netherlands). June 2010.

362. Merck Serono Pharmaceutical Co. (Darmstadt, Germany). June 2010.

363. NanoFormulations '2010 International Conference (Stockholm, Sweden). June 2010.

364. Covidien Surgical Devices and Vascular Therapies Divisions (Cambridge, MA). October 2010.

365. World Lyophilization Summit (Cambridge, MA). May 2011.

366. Alvogen (Cambridge, MA). March 2012.

367. Colgate-Palmolive Co. (Cambridge, MA). April 2012.

368. FDA Parenteral Drug Association Glass Quality Conference (Washington, DC). June 2012.

369. University of Puerto Rico (Humacao, Puerto Rico). January 2013.

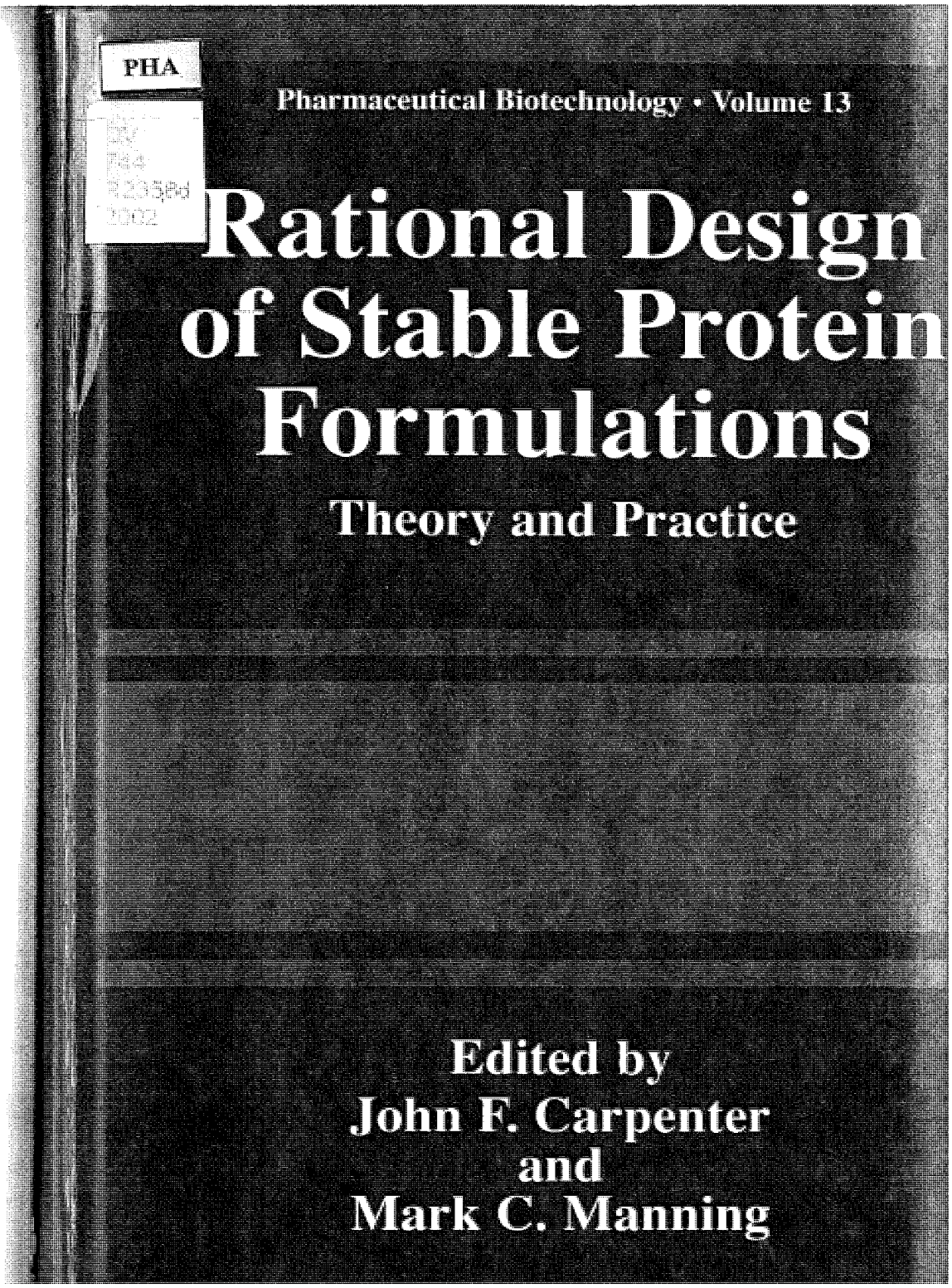
370. International Conference on Creating and Leveraging Intellectual Property in Developing Countries (Durban, South Africa – *via teleconference*). November 2013.

371. Northwestern University (Evanston, IL). April 2015.

Momenta Pharm. v. BMS, IPR215-01537
41/41 BMS EXHIBIT 2016

2520

Samsung Bioepis Exhibit No. 1002
Page 7966



Samsung Bioepis Exhibit No. 1002
Page 7967

Rational Design of Stable Protein Formulations

Theory and Practice

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Page 7968

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Samsung Bioepis Exhibit No. 1002
Page 7969

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Samsung Bioepis Exhibit No. 1002
Page 7970

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Samsung Bioepis Exhibit No. 1002
Page 7972

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Samsung Bioepis Exhibit No. 1002
Page 7973

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Samsung Bioepis Exhibit No. 1002
Page 7974

Contents

Chapter 1

Practical Approaches to Protein Formulation Development
Byeong S. Chang and Susan Hershenson

Introduction 1
Preparation for Formulation Development 3
Resource Requirements for Formulation Development 3
Useful Information for Designing Formulations 4
Preformulation Development 4
Characterization of Protein Pharmaceuticals 5
Accelerated Stability Studies 5
Development of Analytical Methods 6
Evaluation of the Significance of Problems 7
Formulation Development 10
Formulation Options for Protein Pharmaceuticals 10
Typical Protein Stability Problems: Causes and Solutions 13
Optimization of Formulation Variables 13
Necessary Studies for Formulation Development 15
Strategies to Overcome Difficult Formulation Problems 17
Formulation in Commercial Product Development 18
Critical Formulation Decisions During Pharmaceutical Development 18
Formulation for Early Preclinical and Clinical Studies 19
Commercial Formulation 19
Regulatory Issues in Formulation Development 20

Samsung Bioepis Exhibit No. 1002
Page 7975

Appendix: List of Regulatory Documents 22
References 23

Chapter 2

Recombinant Production of Native Proteins from *Escherichia coli*
Tsutomu Arakawa, Tiansheng Li, and Linda O. Narhi

Introduction 27
Distribution of Expressed Proteins 28
Cell Washing and Lysis 32
Purification of Soluble, Folded Proteins 34
Purification and Refolding of Soluble, Misfolded Proteins 35
Purification and Refolding of Proteins from Inclusion Bodies 36
Washing and Solubilization of Inclusion Bodies 36
Purification of Expressed Proteins from Inclusion Bodies 36
Refolding Mechanism 38
Disulfide Bond Formation 41
Removal of Denaturant 41
Effects of Tag Sequences 44
Effects of Excipients 44
Response Surface Methodology 47
High Pressure Disaggregation and Refolding 48
Methods to Analyze Folded Structures 48
Bioactivity 49
Binding to Receptors 49
Dilsulfide Bond Analysis 50
Spectroscopy 50
Conformational Stability 51
Limited Proteolysis 51
References 51

Chapter 3

Physical Stabilization of Proteins in Aqueous Solution
Brent S. Kendrick, Tiansheng Li, and Byeong S. Chang

Introduction 61
Overview of Physical Stability 62

Samsung Bioepis Exhibit No. 1002
Page 7976

Thermodynamic Control of Protein Stability 62
Kinetic Control of Protein Stability 63
Interactions of Excipients with Proteins 65
Preferentially Excluded Cosolvents 66
Buffers/Salts 67
Specific Binding of Ligands 68
Protein Self-Stabilization 69
Physical Factors Affecting Protein Stability 70
Temperature 70
Freeze-Thawing 71
Agitation and Exposure to Denaturing Interfaces 71
Pressure 72
Conclusions 73
Appendix: Derivation of the Wyman Linkage Function and Application to the Timasheff Preferential Exclusion Mechanism 73
References 78

Chapter 4

Effects of Conformation on the Chemical Stability of Pharmaceutically Relevant Polypeptides
Jeffrey D. Meyer, Bert Ho, and Mark C. Manning

Introduction 85
Relationship Between Structure and Deamidation Rates 86
Primary Structure Effects 87
Secondary Structure Effects 89
Tertiary Structure Effects 91
Summary of Structure Effects on Deamidation 92
Role of Structure in Protein Oxidation 92
Types of Oxidation Processes 93
Effects of Oxidation of Surface and Buried Methionines on Protein Structure 95
Limiting Solvent Accessibility of Residues 96
Conformational Control of Oxidation in Aqueous Solution 97
Structural Control of Oxidation in Lyophilized Products 99
Summary of Structural Control of Oxidation 100
Summary 101
References 101

Samsung Bioepis Exhibit No. 1002
Page 7977

Chapter 5

Rational Design of Stable Lyophilized Protein Formulations: Theory and Practice
John F. Carpenter, Beyong S. Chang, William Garzon-Rodriguez, and Theodore W. Randolph

Introduction 109
Minimal Criteria for a Successful Lyophilized Formulation 111
Inhibition of Lyophilization-Induced Protein Unfolding 112
Storage at Temperatures Below Formulation Glass Transition Temperature 113
The Water Content is Relatively Low 114
A Strong, Elegant Cake Structure is Obtained 114
Steps Taken to Minimize Specific Routes of Protein Chemical Degradation 116
Rational Design of Stable Lyophiilized Formulations 117
Choice of Buffer 118
Specific Ligands/pH that Optimizes Thermodynamic Stability of Protein 119
Trehalose or Sucrose to Inhibit Protein Unfolding and Provide Glassy Matrix 120
Bulking Agent (e.g., Mannitol, Glycine or Hydroxyethyl Starch) 126
Nonionic Surfactant to Inhibit Aggregation. 127
Acknowledgments 127
References 127

Chapter 6

Spray-Drying of Proteins
Geoffrey Lee

Introduction: Why Spray-Dry a Protein? 135
Developments in the Last 10 Years 136
The Practice of Spray-Drying Proteins 139
Type of Equipment 139
Spray-Drying Conditions 140
Influence of Formulation 147
Pure Proteins 147
Formulated Systems 149
Use of Added Surface Active Substances 151

Samsung Bioepis Exhibit No. 1002
Page 7978

Concluding Remarks 156
References 156

Chapter 7

Surfactant-Protein Interactions
Theodore W. Randolph and LaToya S. Jones

Introduction 159
Proteins and Surfactants at Surfaces 161
Protein-Surfactant Interactions in Solution 166
Surfactant Effects on Protein Assembly State 167
Surfactant Effects on Proteins During Freezing, Freeze-Drying and Reconstitution 169
Enzymatic Degradation of Non-Ionic Surfactants 170
Recommendations for Protein Formulation 170
References 171

Chapter 8

High Throughput Formulation: Strategies for Rapid Development of Stable Protein Products
Rajiv Nayar and Mark C. Manning

Introduction 177
Overall Structure of the HTF Approach 179
Role of an Established Decision Tree for Formulation Design 181
Constraints on a Pharmaceutically Acceptable Protein Formulation 182
Proper Choice of Dosage Form 183
Preformulation Studies 185
Proper Choice of Excipients 186
Estimates of Resources Needed for Formulation Development 188
Use of Software and Databases to Assist in the HTF Process 189
Essential Analytical Methods 191
Stability Protocols 193
Unified Strategy for HTF 194
References 195

Index 199

Samsung Bioepis Exhibit No. 1002
Page 7979

1

Practical Approaches to Protein Formulation Development

Byeong S. Chang and Susan Hershenson

INTRODUCTION

As is the case with other pharmaceuticals, formulation development is one of the critical steps in developing a protein as a therapeutic product. Development of stable protein formulations may require even more resources and effort than conventional small molecule pharmaceuticals. Proteins typically have more stability issues as a result of their complexity and delicate structural stability. Fortunately, a great deal of research regarding protein stability has been conducted and this information is readily available in the literature (reviewed by Manning et al., 1989; Chen, 1992; Ahern and Manning, 1992a, 1992b; Arakawa et al., 1993; Cleland et al., 1993; Wang and Pearlman, 1993; Pearlman and Wang, 1996; Volkin and Middaugh, 1997). Ultimately, it would be ideal to be able to develop a pure pharmaceutical containing only the native protein. However, it is not practical to have only the native form of a protein in the formulation because the protein must be purified from a complex biological mixture containing a pool of other proteins which includes misfolded, denatured, and degraded forms of the same protein. Furthermore, a major challenge is to maintain the integrity of the purified protein during routine pharmaceutical processing, storage, handling, and delivery to the patient. One could envision achieving this goal by developing a formulation with

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Samsung Bioepis Exhibit No. 1002
Page 7980

perfect stability, i.e., no physical and chemical change in the protein. Because proteins are complex molecules composed of numerous reactive chemical groups and delicate three-dimensional structures, identifying a set of conditions to keep all components stable is virtually impossible. In general, commercial therapeutic protein formulations are developed under the assumption that some degree of physicochemical changes will occur during storage and handling.

Realizing that it is impossible to develop a perfectly stable formulation, especially while meeting an aggressive product development timeline, the main objective then becomes one of maintaining the appropriate safety and efficacy of the product. In order to achieve this objective, it is imperative to understand the broad spectrum of degradation pathways affecting proteins, and to have available equipment and expertise in an extensive repertoire of analytical methods. Formulation development focuses on determining the potential degradation pathways, assessing the significance of each and optimizing variables to minimize the degradation products that are clinically significant.

Regulatory guidelines also are critical elements for guiding formulation development. They provide information about how to conduct studies and obtain useful results for evaluating formulations. The results obtained allow formulation scientists to write an appropriate developmental pharmaceutics section in regulatory filings. The guidelines also help to evaluate the significance of some inevitable degradation products that are produced during manufacturing, shipping and storage. For example, if the degradation products have properties comparable to those of the desired product with respect to activity, efficacy, and safety, they can be classified as product-related substances. This classification is significantly different from considering the degradation product as an impurity when there is not sufficient supporting evidence to justify classification as a product-related substance (Appendix, Regulatory Document 1). Understanding of these practical issues of regulatory requirements is critical for formulation scientists during design, implementation, evaluation and reporting of their studies.

In addition to insight into the scientific and regulatory issues, developing commercial formulations requires a clear understanding of the potential market. For example, indication, patients, method of delivery, frequency of dosing, typical dose requirement, market distribution and other business-related information will provide directions for the design of a successful formulation. Also, it is important to consider the competitiveness of the formulation as compared to other products available in the market.

In this chapter, an overview of critical factors affecting the design of therapeutic protein formulations and a general guide to developing commercially viable dosage forms for protein pharmaceuticals will be discussed. Since the majority of practical issues are not covered very well in the scientific literature, this chapter also includes information from regulatory guidance documents (see Appendix), labels from marketed products and routine industrial experience.

Samsung Bioepis Exhibit No. 1002
Page 7981

PREPARATION FOR FORMULATION DEVELOPMENT

Resource Requirements for Formulation Development

A list of resources that should be available before starting formulation development is summarized in Table 1. Some resources may not be as critical as others, depending on the nature of problems encountered, but it is important to have sufficient resources to discover major formulation issues as early as possible in the product development process. Without the appropriate equipment and personnel, the development of an acceptable formulation can be greatly hampered, potentially to such a degree that the product is never brought to market. Of course, not all of the resources need to be at the company developing the therapeutic protein, but if outside contractors are employed it is essential that timely access to resources is available. In addition, the quality of raw materials should be carefully evaluated because unexpected impurities may introduce unnecessary

Table 1.
Resource Requirements for Initial Protein Formulation Development

Resources	Requirement	Example
Purified protein	Representative of manufacturing process; sufficient quantity to cover dose bracket, formulation variables, and stress conditions; minimum complication by impurity (precipitation of impurity, degradation by impurity like proteolytic cleavage).	Purified bulk, sample from final purification process
Qualified excipients	Pharmaceutically acceptable quality, manufacturers with qualified production procedures and sufficient scale, specifications on critical impurities, quality that can be carried on to clinical studies and commercial distribution.	USP, Ph. Eur, JP
Access to fill finish facility	Capability to sterilize container/closure components; fill/finish under aseptic environment; head-space purge system; drying equipment	Sterile hood, filling machine, lyophilizer
Analytical instruments	Structural analyses; concentration determination, chromatographic analyses; electrophoresis; bioassays; other microcharacterizations.	CD, UV, fluorescence, HPLCs, mass-spectrometry, SDS-PAGE
Facility to accommodate stability studies	Controlled temperature, controlled light exposure, controlled relative humidity, devices to provide controlled agitation.	Freezer, refrigerator, incubator, light chamber, RH incubator, agitator

Samsung Bioepis Exhibit No. 1002
Page 7982

Table 2.
Examples of Information Useful for Designing Formulation Studies

Information	Examples
Clinical indication	Site of treatment (self-administration, office visit, hospital), methods of delivery, concomitant medication, competition.
Patient population	Age, strength, tolerability, capability to manipulate devices, sensitivity to excipient
Typical routes of delivery	Injectables (IV, SC, IM, IP, ICV, IT, IO), topical, inhalation, nasal, oral, etc.
Dose requirement	PK profile, frequency of dosing, variable vs. fixed dose, single-dose/multidose
Drug interaction	Co-administration with other drug, dilution or reconstitution with other solution; presence of undesirable compounds like reducing sugars, preservatives
Typical dosage forms	Liquid, lyophilized, spray-dried, aerosol by liquid or powder, other novel carrier; stability, physical properties, reconstitution art
Container/closure	Vial/stoppers, prefilled syringes, prefilled cartridges, dual-chamber cartridges, blister packages, product contact material, leacheates, breakage, light sensitivity, moisture penetration
Delivery device	Syringes, prefilled-syringes, pen injectors, auto-injectors, needle-free injectors, inhalation devices, infusion pumps

complications in the stability profile that is determined during formulation development and testing.

Useful Information for Designing Formulations

The configuration of a protein formulation is affected by how the drug will be used as a product. If such information is available when designing formulation studies, it is recommended to consider the limitations and challenges associated with each application. Examples of such information are listed in Table 2.

PREFORMULATION DEVELOPMENT

It is important to understand the critical properties of a protein before starting large studies to design and test the final formulation. Preformulation studies are designed to learn about the protein's susceptibility to a variety of pharmaceutically relevant stresses. The main objectives of preformulation research

Samsung Bioepis Exhibit No. 1002
Page 7983

Table 3.
Information Obtained from Pre-formulation Studies for Protein Pharmaceuticals

Characterization	Examples
Physical properties	Primary, secondary, tertiary and quaternary structures, solubility, viscosity, self-association, hydrophobicity, molecular weight, extinction coefficient, glycosylation, effects of ionic strength, etc.
Biological properties	Substrate or receptor affinity, in vitro activity model, *in vivo* preclinical model, etc.

include: general characterization of the product; investigation of potential stability issues; development of relevant analytical methods; establishment of a stability profile with stability-indicating assays; and identification of major formulation challenges. A summary of these issues will be presented in this section.

Characterization of Protein Pharmaceuticals

There is available in the literature extensive coverage of analytical methods and their principles for the characterization of proteins (e.g., Jones, 1993; Reubsaet et al., 1998; Herron et al., 1995). General points to consider when characterizing a protein as an active ingredient are listed in Table 3. As a protein's biological activity is dependent on its structure, significant emphasis has been given to structural properties and stability against various stresses.

Accelerated Stability Studies

In order to predict potential stability problems within a short period of time and to develop appropriate analytical methods, proteins are exposed to stronger-than-real stresses and various degradation products induced by the stresses are examined. The results obtained from these so-called "accelerated stability studies" might also be useful to predict the kinetics of the degradation processes under real handling conditions, when there are not sufficient real-time results available because of time and resource constraints. However, the accelerated stability study is not acceptable to determine expiry of the product, so it is best used to rank order the importance of different degradation pathways. Approaches and cautions in the extrapolation of data from accelerated stability testing to real-time stability and normal handling conditions are discussed in detail below.

Samsung Bioepis Exhibit No. 1002
Page 7984

Table 4.
Various Conditions Used to Accelerate Protein Degradation

Stresses	Routine ranges	Practical applications	Problems to monitor
Temperature	0–50°C	Storage, shipping, handling, delivery	Structural changes (precipitation, aggregation, recovery loss), solubility, increased reaction rates for all degradations
Light	>1.2 million lux hrs illumination, >200 watt hrs/square meter UV energy	Light exposure, container, package	Oxidation, cleavage
Freezing	Multiple freeze-thaw, liquid nitrogen freeze	Frozen storage, accidental freezing, lyophilization	Precipitation, aggregation, pH change, crystallization of excipients
Oxidation	Oxygen purge, peroxide spike	Storage, excipient stability, impurity	Oxidations, inactivation
Humidity	0–100% RH	Storage, container integrity, powder	Moisture content, moisture related degradations
Mechanical stresses	Vortex, agitation, shear-stress ($3000\,s^{-1}$)	Manufacturing, filling, shipping, handling, delivery	Precipitation, aggregation, recovery loss
Other denaturants	—	Impurities, pH, denaturing excipients	Precipitation, aggregation, recovery loss, structural changes

Conditions to accelerate various degradation reactions in protein products and potential problems to monitor are listed in Table 4. Proteins contain numerous amino acid side chains and delicate three-dimensional structures, which can be susceptible to different stresses. Therefore, it is important to test the protein under a variety of physical and chemical stresses in order to provide a good simulation of the degradation products that can be generated.

Development of Analytical Methods

A brief summary of typical analytical methods is presented in Table 5. It is essential to have a wide range of analytical methods available to identify and characterize degradation products. The analytical methods should be further selected and customized to accommodate the specific needs for each protein product.

Samsung Bioepis Exhibit No. 1002
Page 7985

Table 5.
Typical Methods Used to Characterize Proteins and Degradation Products

Methods	Examples	Applications
Column chromatography	HPLC, FPLC, low pressure LC; size-exclusion, reversed-phase, ion-exchange, hydrophobic, affinity columns; coupled with UV, fluorescence, RI, and other analytical instruments as detectors	Most physical and chemical degradations, excipient impurities, leacheates
Electrophoresis	SDS-PAGE, native PAGE, isoelectric focusing, capillary electrophoresis, etc.	Degradations with changes in size and/or charge
Spectroscopy	CD, fluorescence, FTIR, UV, Raman, NMR, etc.	Structural changes, chemical modifications of side groups
Thermal analysis	Differential scanning calorimetry, thermogravimetric analysis, thermomechanical analysis, etc.	Protein structure, lyophilized cake structure, powder characterizations
Light scattering/turbidity	Dynamic light scattering, other light scattering devices, turbidity, particle size determination, particle counter, etc.	Aggregation, precipitation, molecular weight determination
Other microcharacterization methods	Peptide mapping, peptide sequencing, amino acid analysis, mass spectrometry, other specific analyses for individual reactive groups	Identification of impurities and chemical degradation, analysis of complex proteins, e.g., antibody and glycoprotein

Evaluation of the Significance of Problems

As stated earlier, it would be ideal to have a pure protein in an absolutely stable formulation. In reality, however, scientists have to design formulation based on compromises that deal with several different potential problems. To make matters worse, the formulation needs to be recommended long before it is evaluated fully, because of typical aggressive timelines in the industry. In general, the formulation will have to be optimized based on assumptions and extrapolations of results obtained during a limited time. Therefore, it is important to utilize the given time efficiently, by collecting as much relevant information as possible for evaluating the significance of each problem.

Due to the marginal stability of proteins, it is possible to create rapidly a variety of degradation products during accelerated stability testing. However, not

Samsung Bioepis Exhibit No. 1002
Page 7986

all the degradation products that are observed will be significant under normal handling, shipping and storage conditions. Furthermore, the rank order of different degradation processes under accelerated stability testing will NOT be the same under practical handling conditions, because each reaction has a different temperature dependency, i.e., reaction order and activation energy. Another important thing to keep in mind when evaluating different degradation processes is the contribution to the pharmaceutical quality of the product. Critical degradation products should be designated not on the quantity obtained during accelerated stability studies, but on a comprehensive understanding of their contributions to the quality of the product.

Quantitative Assessment. Usually, the rate of each degradation reaction during real storage conditions (e.g., at 4–8°C) should be very slow. In order to predict the rate under real storage conditions, reaction rates obtained under accelerated stability conditions can be extrapolated by using the Arrhenius equation.

Predicting the correct reaction rate requires a proper understanding of the reaction order, because the amount of the degradation product does not linearly increase over time unless the reaction follows zero-order kinetics. One way to determine the reaction order is to calculate the linearity between the concentration of residual native protein and time. The reaction order can be calculated from:

$$dC/dt = kC^n \tag{1}$$

where, C is the protein concentration, t is the time, k is the reaction rate constant, n is the reaction order. For a zero order reaction $n = 0$ and $C_0 - C = k \cdot t$. For a first order reaction $n = 1$ and $\log C_0 - \log C = k \cdot t$. For a second order reaction $n = 2$ and $1/C - 1/C_0 = k \cdot t$. For each reaction order a characteristic transformation of concentration will show a linear relationship with time, e.g., log C will show a linear relationship with time for a first order reaction, whereas $1/C_0$ will be linear versus time for second order reactions.

After obtaining the reaction rate constants for different temperatures, the activation energy can be found by using with the Arrhenius equation.

$$k = A \cdot e^{(-Ea/RT)} \tag{2}$$

The activation energy is obtained by plotting (log k) vs (1/T) and determining the slope of the plot. Using the activation energy, the reaction rate constant for real storage condition, e.g., 2–8°C for refrigerated storage, can be estimated by extrapolation.

Care must be taken when extrapolating the rate constants because there are numerous cases when the Arrhenius relationship does not apply. The Arrhenius reaction applies only to irreversible reactions where the product is accumulated as a single quantifiable species. If the degradation product is the result of serial

Samsung Bioepis Exhibit No. 1002
Page 7987

reactions, the reaction order and rate constant will be determined only by the rate-limiting reaction at the testing condition. If the rate-limiting reaction changes or another significant variable is introduced at the extrapolated condition, then the extrapolated rate constant will be in error. Such complications routinely can be found in various degradation pathways that are affected by temperature-sensitive reactions such as structural changes, pH changes, by physical changes such as the glass transition of an amorphous phase in a lyophilized formulation and by changes in reactants like dissolved oxygen. For example, proteins have relatively high activation energies for structural changes. Thus, many reactions that are dependent on a major perturbation of the native protein structure tend to cause substantial damage under accelerated storage conditions, e.g., higher temperature. However, many of these reactions are not necessarily major problems when the product is stored at 2–8°C where the protein maintains its native conformation. In contrast, degradation reactions with lower activation energies, which might not be coupled to protein conformational changes (e.g., oxidation of surface methionine residues), tend to be much more problematic in the development of protein formulations.

Results from stresses (e.g., agitation) other than heat imposed in accelerated stability studies potentially could be extrapolated in a similar way to storage and actual handing conditions. The major challenge is the ability to assess quantitatively the magnitude of the stress so that the stress-stability relationship can be established. To date there are not published guidelines for such quantitation.

Qualitative Assessment. Not all of the degradation products are equivalent in terms of their contribution to the pharmaceutical quality of the protein product. Therefore, a qualitative assessment of each degradation product is important when weighing their significance. Regardless of their quantity, some degradation products are generally less acceptable than others. If the degradation product comprises the safety of the product, then it should be considered less acceptable. For example, even at levels of a few percent or less of the total protein population, non-native aggregates can cause adverse reactions in patients such as immune responses and even anaphylactic shock. Also, if the degradation reaction results in the inactivation of protein, it needs to be considered more important than other degradation reactions that do not affect the activity. For example, certain chemical changes (e.g., deamidation) may not alter the activity of a given product, whereas other reactions (e.g., oxidation) may render that product inactive. The relative impact of each degradation product on safety and efficacy cannot be predicted and must be determined for each protein therapeutic.

Information useful for the qualitative evaluation of degradation products includes the identity, clinical and preclinical experiences with the degradation product, biological activity, stability and potential side effects. Regulatory documents also provide clear guidance about what assessment is necessary for protein degradation products (Appendix, Regulatory Documents 2,3). The criteria for

Samsung Bioepis Exhibit No. 1002
Page 7988

identification, reporting and qualification of degradation products are set up based on the total patient exposure and percentage of the degradation products (Appendix, Regulatory Document 3). When certain degradation products cannot be prevented from forming in the formulation, their inclusion in the product must be qualified, which may require that additional information on safety and efficacy be obtained as recommended in the regulatory guidelines (Appendix, Regulatory Documents 2,3). Obtaining this information requires a significant amount of additional work, which may include clinical studies.

FORMULATION DEVELOPMENT

As discussed above, the critical parameters affecting the pharmaceutical quality of a protein therapeutic are defined during the preformulation studies. In formulation development, the effects of formulation variables on the defined critical parameters are examined to optimize protein stability. Which variables are most important depends of the formulation type chosen. For example, resistance to agitation and/or accidental freezing is a critical property for an aqueous formulation, which would not be a concern for a lyophilized formulation. Similarly, choosing excipients that provide a glassy matrix to stabilize the protein is only a concern for dried formulations.

Formulation Options for Protein Pharmaceuticals

Different types of formulations need to be developed based on clinical needs, patient compliance, delivery method, stability of the drug, storage and distribution, and market competitiveness. Having a clear plan about what type of formulation is desired will allow one to design better formulation studies. Liquid formulations have been generally preferred due to the convenience of manufacturing and use. However, protein drugs may not be stable enough to be handled as a liquid formulation. Dried formulations (e.g., lyophilized) or suspension formulations (e.g., insulin zinc suspension) have been successfully used to overcome stability problems. In addition, specific applications and delivery may demand the appropriate type of formulation, e.g., a spray-dried powder for pulmonary delivery. As details of the stability issues are discussed in other chapters in this book, only practical issues regarding these different types of formulations will be summarized here.

Liquid Formulations. It is important to understand that developing conditions to keep proteins stable in a liquid form for a pharmaceutically relevant

Samsung Bioepis Exhibit No. 1002
Page 7989

storage time (e.g., two years) is not a simple task. For most proteins, including relatively stable ones, at least some degradation should be expected even during refrigerated storage. Unless there is strong evidence supporting that the protein remains stable for two years and the evidence is supported by a broad spectrum of analytical methods, one needs to be very careful about the final decision to market a liquid formulation. Physical or chemical changes that have low activation energy should be monitored especially carefully under real storage conditions. If there is a degradation product that can significantly affect the pharmaceutical quality at its minimum concentrations (e.g., formation of particulates) great care must be taken to assure that such a product does reach unacceptable levels during real-time storage. If long-term storage studies are not implemented until late in the product development process, there is a risk that problems due to unacceptable levels of degradation products will not be discovered in time to test alternative formulations. In addition to stability during storage, temporary exposure to temperatures outside the recommended conditions should also be tested because it can affect the quality of the drug. Also, results obtained from multiple freeze-thawing cycles will be useful to determine if the formulation can accommodate unexpected freezing during distribution and storage. Likewise, sensitivity to agitation or surface denaturation needs to be understood to support formulation choices as well as shipping and handling guidelines. For formulations containing low protein concentration, special attention is required to avoid impurity-related stability issues (e.g., oxidation fostered by metal contaminants in excipients).

Solid Dosage Forms. Recent improvements in devices designed for easier use of lyophilized products, e.g., dual chamber syringes, dual chamber cartridges and convenient reconstitution devices, have helped pharmaceutical industries to develop lyophilized products without too many concerns surrounding patient compliance issues. Because lyophilized products have less stability-related issues and have a much greater potential tolerance for room-temperature storage, many biopharmaceutical industries consider the lyophilized formulation as a default option. In fact, lyophilization has been widely utilized to overcome various stability issues of labile proteins. However, it is important to note that lyophilization can present its own challenges, particularly in designing appropriate formulations and economic cycles. Detailed discussion about the rational design of stable lyophilized formulations is available in another chapter in this book.

In addition to the required stability, a successful lyophilized formulation should also have the desired physical properties of the dried powder, e.g., ruggedness of the cake and the maintenance of the physical states for each of the ingredients. For example, crystallization of an initially amorphous excipient during storage can lead to unacceptable degradation of the protein product and loss of cake structure. Furthermore, optimal water content for protein stability should be studied and controlled during storage. It is preferable to define an acceptable

Samsung Bioepis Exhibit No. 1002
Page 7990

range of water contents to provide flexibility in the manufacturing process. To assure that the appropriate water content is maintain, the integrity of container/packaging should be monitored in a high relative humidity environment.

The identity and volume of the reconstitution medium should also be clearly defined, because the use of injectable fluids other than the recommended one may compromise the quality of the product (e.g., solution tonicity or protein stability). Reconstitution should be convenient and rapid, and the stability of the reconstituted solution during handling and delivery needs to be demonstrated.

Only recently have spray-dried formulations become commonly used with the advent of novel protein delivery technologies. For example, spray-dried powders have been useful for developing products that require uniform particle size (e.g., pulmonary inhalation for systemic delivery). Similar issues to the lyophilized formulation apply to the spray-dried formulation, and the details of development of spray-dried formulations are presented in another chapter in this book. In addition to protein stability, physical properties pertinent to powder processing and powder stability (e.g., flowablity, hygroscopicity, agglomeration, density and crystallization of excipients) need to be well characterized.

Single Dose and Multidose Forms. Most protein pharmaceuticals are marketed as single dose forms. However, multidose formulations are useful when the dose needs to be split (e.g., dose titration or dose combination). Multidose formulations are distinguished by the presence of preservatives in the formulation, which prevent microbial contamination and/or growth during multiple disruptions of container closure integrity. In general, the addition of preservative(s), regardless of the preservative used, significantly changes the stability profiles of proteins (Maa and Hsu, 1996; Fransson et al., 1997; Lam et al., 1997). In some extreme cases, visible precipitation and aggregation have been reported. Therefore, the effect of various preservatives on the stability of protein should be carefully examined. Other experiments required to qualify a multidose formulations include the preservative effectiveness test and the stopper self-sealing test. Detailed procedures and specification are available from the USP (U.S. Pharmacopeia), the Ph. Eur. (European Pharmacopeia) and the JP (Japan Pharmacopeia).

Desired properties of the ideal preservative include: effectiveness at low concentration against a wide variety of organisms; chemical stability; solubility; compatibility with the protein drug, excipients and auxiliary agents; free from objectionable odor, taste, color and stinging; and non-toxic and non-sensitizing both internally and externally at the required concentration. Also, it must not absorb, penetrate, or interact with containers or closures (Thompson, 1998). For further details on preservatives, readers are referred to Regulatory Document 4 in the Appendix and Thompson (1988). Examples of typical preservatives used for parenteral protein pharmaceuticals are benzyl alcohol, phenol, *m*-cresol and benzalkonium chloride.

Samsung Bioepis Exhibit No. 1002
Page 7991

Typical Protein Stability Problems: Causes and Solutions

Table 6 summarizes typical stability problems observed during protein formulation development and potential methods to solve each problem. The list does not represent the complexity of multiple problems that can be experienced with a given protein. Formulation research should be designed to handle each protein based on its unique stability profile.

Optimization of Formulation Variables

Overview of the Process. The optimization of formulation variables for product stability is the most critical part of protein formulation development. Various formulation excipients and buffers (Table 7) can be utilized and must, therefore, be chosen to maximize the pharmaceutical quality of the product (i.e., stability and activity) without introducing significant side effects. Among the listed formulation variables, the most powerful one is pH. Problems associated with the physical properties of a protein, e.g., precipitation due to solubility and/or stability, are generally very difficult to manage by other formulation means. Optimization of pH is a simple but very useful solution for such problems (Kolvenbach et al., 1997). Most chemical reactions also are affected by pH, e.g.,

Table 6.
Typical Stability Problems Observed in Protein Pharmaceuticals

Problems	Potential causes	Possible solutions
Non-covalent aggregation	Solubility, structural changes, heat, shear, surface, denaturants, impurities	pH, ionic additives, amino acids, surfactants, protein concentration, raw material purity
Covalent aggregation	Disulfide scrambling, other unknown mechanisms	pH, inhibit non-covalent aggregation
Deamidation	pH < 5.0 or pH > 6.0	pH optimization
Cyclic imide	pH around 5	pH optimization
Cleavages	Protease impurity, other unknown mechanisms	pH, product purity, inhibitors
Oxidation	Active oxygen species, free radicals, metals, light, impurity	Excipient purity, free-radical scavenger, active oxygen scavengers
Surface denaturation, adsorption	Low protein concentration, specific affinity, protein hydrophobicity	Surfactants, protein concentration, pH

Samsung Bioepis Exhibit No. 1002
Page 7992

Table 7.
Important Components of Protein Formulations

Formulation Variables	Desired attributes	Examples
pH	Provides good physical properties of protein, minimize degradations	
Stabilizer	Inhibit degradations, effective at low concentrations	Surfactants, sugars, salts, antioxidants
Solubilizer	Improve the solubility, effective at low concentrations	Salts, amino acids, surfactants
Buffer	Good buffering capacity, stable to temperature change, stable to freezing, good safety record	Phosphate, acetate, histidine, glutamate
Tonicity modifier; bulking agent	Inert, good safety record	Sodium chloride, sorbitol, mannitol, glycine

deamidation, cyclic imide formation, disulfide scrambling, peptide bond cleavage, and oxidation (see reviews listed above). Other functional excipients should be also carefully evaluated for the benefit of the product (e.g., use of sucrose to stabilize protein during lyophilization and storage in the dried solid).

In addition to their intended use, excipient candidates should be also qualified as appropriate pharmaceutical ingredients. Generally, it is preferred to select excipients that have been used in marketed products with a relevant route of delivery. Further, it is preferable if these excipients have been used with similar frequency of dosing, history of chronic use and similar patient populations. Otherwise, the approval and safety of the excipients need to be carefully examined. If the excipient is considered safe with a solid scientific basis or has a proven clinical safety record, it can be considered equivalent to approved excipients. The list of excipients used for parenteral pharmaceuticals is available in the literature (Powell et al., 1998; Nema et al., 1997; Appendix Regulatory Document 5). When it is necessary to introduce other excipients with minimum safety records, a significant risk associated with the excipients will be added to the product development and additional pre-clinical and clinical studies may be needed.

Another important requirement in qualifying an excipient is the purity of the raw material. Depending on the historical use as a pharmaceutical ingredient, several different pharmaceutical grades are available, e.g., USP (U.S. Pharmacopeia), Ph. Eur. (European Pharmacopeia) and JP (Japan Pharmacopeia). These pharmaceutical grade materials should be considered as a primary resource, but the quality provided may not be good enough for specific product development. For example, significant stability problems can be found with some impurities

Samsung Bioepis Exhibit No. 1002
Page 7993

even at concentrations below their specification, e.g., metal ions, peroxides, proteases and reducing sugars. These problems are more prominent in low protein concentration products due to a high impurity-protein ratio, although problems like visible precipitation of the protein may be independent of protein concentration. If adjustment in the existing specification is necessary, it is critical to look into the availability of GMP quality raw materials with modified specifications as early as possible, once the potential problem is identified.

The use of excipients derived from animals (e.g., Tweens) or humans (e.g., human serum albumin) should be avoided if possible due to the risk associated with transmissible diseases like bovine spongiform encephalopathy, Creutzfeldt-Jakob Disease, hepatitis virus and HIV. Numerous regulatory guidelines have been issued to discourage the use of animal/human-derived excipients (Appendix Regulatory Documents 6,7,8). When animal-derived excipients have to be included in the product, the manufacturer will need to demonstrate that their selection is fully justified.

Design of the Study. A design for the formulation optimization study should be in place before commencing the work. A typical study protocol will include the following information:

- Study title
- Study objective
- Source and quality of drug substance and excipients
- Material preparation
- Formulation matrix
  - Formulation variables
  - Protein concentrations or bracket
- Analytical methods
- Storage conditions (temperature, light, humidity)
- Additional sample handling conditions (temporary exposure to stresses, container orientation, etc.)
- Sampling schedule and expected duration of the study
- Plan for data analysis and report

The study can be more efficiently designed by utilizing experimental design software packages, which will help to minimize the resources required for sample preparation, analyses, and data analysis.

Necessary Studies for Formulation Development

Storage Stability Study. Documenting that the formulation will keep the protein stable until the desired expiry can be the most time-consuming part of

Samsung Bioepis Exhibit No. 1002
Page 7994

formulation development. The expiry requirements are determined by distribution not regulatory requirements. One year is probably too short for effective manufacturing and distribution through normal channels. In general, a shelf life of 18 month is considered acceptable for commercialization. Results obtained from accelerated stability studies are useful for predicting potential degradation products and appropriate analytical methods, but whatever the actual shelf life, it must be supported by sufficient real-time storage data to obtain regulatory approval. Thus, it is important to establish a final formulation and start the real-time storage studies as early as possible during product development.

Process Development. Formulations that can be prepared on a small scale without experiencing any problems may encounter significant problems during the scale-up of the process. For example, mixing solutions in a large stainless steel tank, pumping solutions through stainless steel tubing, filtration and filling through a high-speed filling machine can introduce unexpected stresses to the protein. An increase in the formation of particulates, along with a loss of proteins due to surface adsorption and aggregation, has routinely been observed. It is important to expose the formulation to equivalent stresses and make sure that no formulation adjustment is necessary to accommodate the manufacturing processes. Again, this testing should be done as early in the development process as possible.

Transportation, Handling and Delivery Study. Unexpected environmental changes can be encountered during the distribution and handling of products (e.g., accidental freezing, exposure to temperatures different from the recommended conditions, vigorous agitation, etc). It is critical to develop the formulation with these stresses in mind because they can compromise the quality of the product.

Also, during administration to the patient, proteins can be exposed to different types of stresses introduced by the device and the routes of delivery. Examples include incompatibility with the delivery device (e.g., protein aggregation induced by exposure to tubing surfaces) and/or concomitant medication (e.g., protein aggregation induced by co-administered antibiotics).

Preclinical and Clinical Studies. Results documenting the maintenance of the biophysical and biochemical properties of the protein are essential for a final formulation decision. Before finalizing the formulation, it is also important to confirm that it does not affect critical *in vivo* biological properties of the protein (e.g., activity, pharmacokinetic profile, and toxicity profile). Maintenance of a protein's biophysical properties can be examined using various structural analyses (e.g., circular dichroism, fluorescence and infrared spectroscopies, etc.). It is possible to determine the biochemical equivalence of the protein pharmaceutical by *in vitro* activity and/or preclinical *in vivo* bioassays. Results supporting the toxicity profile of the formulation can be generated by both preclinical and clinical studies.

Samsung Bioepis Exhibit No. 1002
Page 7995

Strategies to Overcome Difficult Formulation Problems

Occasionally, problems are encountered that are difficult to overcome with conventional formulation approaches. It is possible to conduct additional studies to confirm that the problem is pharmaceutically acceptable. On the other hand, unique formulation approaches can be introduced to address specific problems. Examples of this approach are provided below.

Qualification of Degradation Products. If decreasing the amount of degradation product below the specified threshold is not feasible, then the degradation product can be defined as a drug substance by demonstrating that it does not affect the pharmaceutical quality of the drug product, i.e., safety and efficacy (Appendix, Regulatory Document 1). If there is not sufficient information available, additional qualification studies recommended in the regulatory guidance can be carried out (see the discussion above). The experience of having these degradation products in the materials used for clinical studies can sometimes be very useful because the results obtained can provide useful insight into the clinical implication of the degradation products.

Site-directed Mutagenesis to Improve Properties. Some problems related to the intrinsic properties of a protein cannot be overcome unless a change in the sequence is introduced. After careful research is carried out to identify the problematic region or residue, the protein can be engineered for better physical and/or chemical properties. For serious problems like precipitation due to insolubility or poor stability, changes in protein sequence have been proven effective for improving physical properties (Murby et al., 1995; Roig and Kennedy, 1995).

Chemical Modifications. The physical properties of proteins can be improved by modifying problematic amino acid side chains with small compounds or large polymers (e.g., attachment of polyethylene glycol) (Fagain, 1995; Guerra et al., 1998; Francis et al., 1998). In addition, there are other desirable improvements that can be achieved by the conjugation chemistry (e.g., enhanced pharmacokinetic profiles, reduced immunogenecity, enhanced adsorption, etc.).

Unconventional Dosage Forms. Some protein stability problems can be resolved by introducing novel approaches in the formulation. Examples include suspension formulations (Defelippis et al., 1998), microencapsulation with cyclodextrin (Brewster et al., 1991), suspension of dry-powder in non-aqueous vehicle (Knepp et al., 1998), and use of non-aqueous vehicles with hydrophobic ion-pairing (Manning et al., 1995).

Samsung Bioepis Exhibit No. 1002
Page 7996

FORMULATION IN COMMERCIAL PRODUCT DEVELOPMENT

Critical Formulation Decisions During Pharmaceutical Development

A brief summary of the important phases of a commercial product development is shown in Figure 1. Ideally, a stable formulation would be available during the early discovery research period to ensure that the protein is in its active and stable form during critical feasibility studies. However, development of a stable formulation for each drug candidate is not practically possible because such an approach would take too much time and too many resources. Generally, formulation development starts after the decision is made to start clinical trials. A

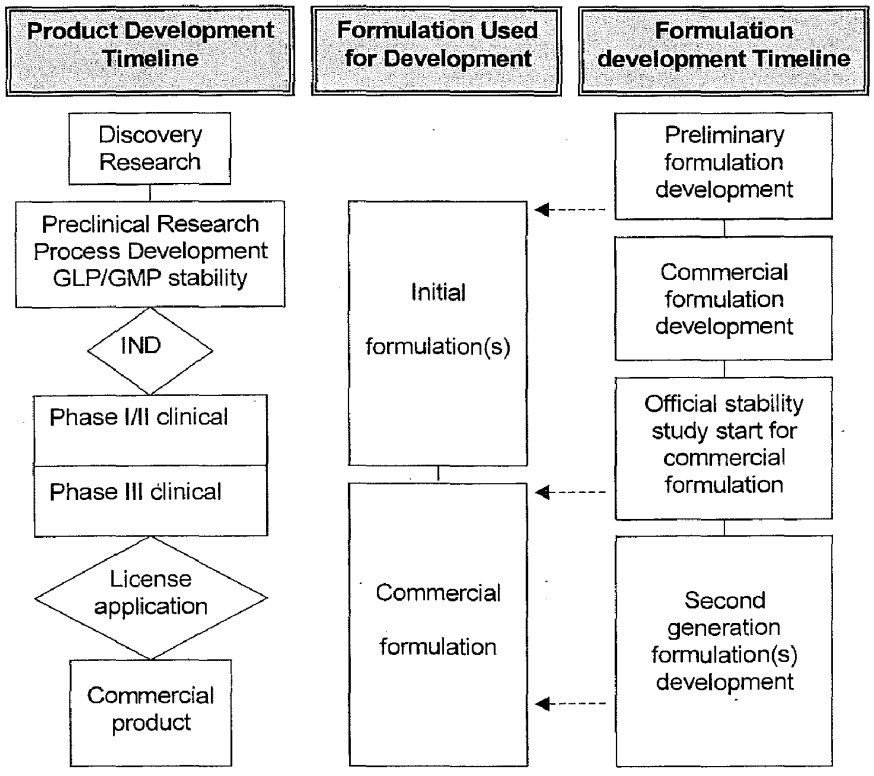


Figure 1. Diagram showing commercial formulation development process.

Samsung Bioepis Exhibit No. 1002
Page 7997

reliable formulation is required to support various preclinical and clinical studies. The formulation can be further improved later to satisfy the clinical, marketing, and regulatory needs. Although formulation development does not necessarily appear rate-limiting during this improvement period, it is important to understand that changing a formulation may require much more supporting work, including additional clinical trials, which can take years.

Formulation for Early Preclinical and Clinical Studies

When a promising protein drug candidate is identified from preliminary feasibility studies, a decision will be made to introduce the drug to clinical studies. In order to obtain regulatory approval to initiate the clinical trials, it is required to demonstrate safety, manufacturing capability, stability of the drug and reproducibility in studying the drug. A sufficiently stable formulation is necessary at this stage because it is important to maintain the quantity and quality of the protein for all research studies from which results will be used for registration of the product with regulatory agencies. Therefore, formulation development becomes a rate-limiting process at this early stage. Formulations can be stored frozen or lyophilized for this purpose. At this stage in product development, the shelf-life requirement is determined by the logistics of supplying drug for clinical trials.

Commercial Formulation

Commercially viable and market competitive formulations have some common features. Most of all, the formulation should maintain the safety and efficacy profile of the protein drug during all the handling and uses specified on the label. Since commercial distribution channels are not equipped for frozen products, shipping and storage at refrigerated temperature or higher are required. Sufficient shelf life needs to be determined under conditions to which the product will be exposed in the commercial distribution system. Various systematic studies need to be carried out to comply with regulatory requirements for registration. It takes 1–2 years to collect all of these results, so the commercial formulation is developed while preclinical studies or early clinical trials (with the preliminary formulation) are in progress. If possible, the commercial formulation should be introduced before the pivotal clinical trial because clinical experience is the most effective way to confirm the safety and efficacy aspects of the formulation. In addition, formulation changes after this point may introduce formidable challenges to the clinical program and to obtaining regulatory approval.

Samsung Bioepis Exhibit No. 1002
Page 7998

Regulatory Issues in Formulation Development

Incorporating regulatory guidelines into the formulation development is not only useful to develop high quality pharmaceutical formulations for maximum benefit to the patients, but also critical to prepare complete documents necessary for regulatory approval for commercialization of the drug. Readers who wish to obtain a comprehensive understanding of the guidelines are referred to the relevant documents published by regulatory agencies. In this section, a brief summary of the guidelines related to the formulation issues is provided.

Guidelines for Stability Studies. Comprehensive guidelines can be obtained from regulatory agencies (Appendix, Regulatory Documents 9–12) and review articles in the literature (Grimm, 1998; Kommanaboyina and Rhodes, 1999; Matthews, 1999). These guidelines generally cover the formal stability studies for bulk drug material, in-process samples and final formulated product. Although some issues may not be relevant for the earlier formulation development work, these guidelines provide key elements for designing stability studies. The guidelines provide information such as what types of experiments should be included in the study protocol, how to propose a stability-indicating profile, and what analytical results are needed to define the purity of the product and the molecular characteristics of degradation products. Another important piece of information provided in the guidelines is the definition of proper experimental conditions, e.g., definition of storage temperature, humidity, light strength, and accelerated and stressed conditions. The guidelines also provide the official protocols to evaluate the results obtained from stability studies.

Results Required to Apply for Regulatory License for a Drug Product. Detailed information regarding the formulation is presented at the Development Pharmaceutics section in the CMC section of regulatory applications (Appendix, Regulatory Docoments 13–16). All of the essential information required to apply for regulatory approval of protein formulations can be found in Note for Guidance on Development Pharmaceutics (Appendix Regulatory Document 17) and Development of Pharmaceutics for Biotechnological and Biological Products (Appendix, Regulatory Document 8). It is important to note that this is currently a European requirement although the international harmonization process may eventually introduce it to U.S. regulatory requirements. A brief summary of the important formulation information is shown in Table 8.

Results Required to File Formulation Amendments. A formulation change can introduce substantial potential adverse effects on the identity, strength, quality, purity or potency of the product as related to the safety or effectiveness of the product. For this reason, it is required that a supplement to the approved license application be submitted. This supplement should include a detailed description of the proposed change. It should also include methods and results for the studies performed to evaluate the effect of the change on the

Samsung Bioepis Exhibit No. 1002
Page 7999

Table 8.
Summary of Information Included in Regulatory Applications

	General information	Comments specific to proteins
Active substance (protein)	Compatibility with excipients and other combined products, and physicochemical characteristics	Structural elements responsible for the biological activity; good coverage of degradations occurring during storage, manufacturing, and delivery.
Excipients	Function of each excipients, justification for their inclusion, and compatibility with other excipients, choice of quality	
Formulated products	Overage, physicochemical parameters, components with appropriate results supporting intended purpose; compatibility with diluent, device, and other drugs in contact before delivery; critical physical properties	Stability of structure in terms of biological activity; formulation optimization for both manufacturing process and stability
Packaging material	Integrity of container and closure during storage, reconstitution, admixture, dilution; sorption to container, leaching, and dose reproducibility	Adsorption, denaturation at the interface, and aggregation of the surface-denatured protein; reconstitution of dry-powder formulation
Manufacturing process	Manufacturing process for the preparation of formulation and its justification, appropriate method of sterilization and justification	Results to support the quality and stability of the protein during the manufacturing process; membrane filtration under aseptic conditions sufficient

product's identity, strength, quality, purity, and potency of the product, as related to the product's safety or effectiveness (Appendix, Regulatory Documents 18–20). When the degradation profile is changed qualitatively or quantitatively, it is recommended to follow the impurity-related guidelines discussed above (Appendix, Regulatory Documents 2,3). The manufacturer must obtain approval of the supplement by FDA prior to distribution of the product made using the change. In general, the following studies (Table 9) can be carried out to confirm

Table 9.
Studies Needed to Support Change in Formulation

Research requirements	Supporting results
Purity	Stability, compatibility, Structural analyses
Potency	*In vitro*/*in vivo* bioassays, preclinical and/or clinical pharmacokinetic comparability , clinical efficacy
Safety	Preclinical safety, clinical safety

Samsung Bioepis Exhibit No. 1002
Page 8000

that the change in the formulation will not affect the safety and effectiveness c the product. It is recommended to consult the regulatory representative to fin out how much additional information may be needed for the approval of th changed formulation.

APPENDIX: LIST OF REGULATORY DOCUMENTS

1. International Conference on Harmonization: Guidance on specifications: Te procedures and acceptance criteria for Biotechnological/Biological Produc Federal Register: August 18, Volume 64, Number 159, pp. 44928–449 (1999).
2. International Conference on Harmonization: Impurities in New Drug Prc ucts. FDA Q3B (11/97).
3. Guidance for Industry. ANDAs: Impurities in Drug Products. Draft guidan FDA (12/98).
4. Note for Guidance on Inclusion of Antioxidants and Antimicrobial Pres vatives in Medicinal Products (CPMP/CVMP/QWP/115/95) EMEA (7/9
5. Inactive ingredient guide: inactive ingredients for currently marketed dı products. (1996) FOI Services, Inc. Rockville, MD.
6. Note for guidance on minimizing the risk of transmitting animal spongifc encephalopathy agents via medicinal products. EMEA (4/99).
7. Note for guidance on plasma-derived medicinal products. EMEA (7/98).
8. Development Pharmaceutics for Biotechnological and Biological Produ Annex to Note for Guidance on Development Pharmaceutics (CPM QWP/155/96). EMEA (10/1999).
9. International Conference on Harmonization: Stability testing of new d substances and products. Federal Register, Sept. 22, Volume 59, Number 1 pp. 48754–48759 (1994).
10. International Conference on Harmonization: Final guideline on stab testing of biotechnological/biological products. Federal Register, July Volume 61, Number133, pp. 36466–36469 (1996).
11. Guideline for Industry: Stability testing for drug substances and drug p ucts: draft guidances. FDA, (6/98).
12. International Conference on Harmonization: Guidelines for the photost ity testing of new drug substances and products. Federal Register, May Volume 62, Number. 95, pp. 27115–2712 (1997).
13. Guideline for Submitting Documentation for the Stability of Human D and Biologics FDA. (2/87).
14. Guidance for Industry. For the Submission of Chemistry, Manufacturing Controls and Establishment Description Information for Human Pla

Samsung Bioepis Exhibit No. 1002
Page 8001

Derived Biological Products, Animal Plasma or Serum-Derived Products. FDA (2/99).
15. Guidance for Industry. INDs for Phase 2 and 3 Studies of Drugs, Including Specified Therapeutic Biotechnology-Derived Products Chemistry, Manufacturing, and Controls Content and Format (Draft guidance) FDA (2/99).
16. Content and Format of Investigational New Drug Applications (INDs) for Phase 1 Studies of Drugs, Including Well-Characterized, Therapeutic, Biotechnology-Derived Products, FDA (11/95).
17. Note for Guidance on Development of Pharmaceutics (CPMP/BWP/328/99). EMEA (01/1998).
18. Demonstration of Comparability of Human Biological Products, Including Therapeutic Biotechnology-Derived Products, April, 1996.
19. Guidance for Industry: Changes to an Approved Application. For Specified Biotechnology and Specified Synthetic Biological Products, 21 CFR 601.12, 314.70; July 24, 1997, Vol 62. No. 142.
20. Guidance for Industry: Changes to an Approved Application: Specified Biotechnology and Specified Synthetic Biological Products; July 1997.

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Samsung Bioepis Exhibit No. 1002
Page 8002

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Samsung Bioepis Exhibit No. 1002
Page 8003

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Samsung Bioepis Exhibit No. 1002
Page 8004

5

Rational Design of Stable Lyophilized Protein Formulations: Theory and Practice

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INTRODUCTION

For ease of preparation and cost containment by the manufacturer, and ease of handling by the end user, an aqueous therapeutic protein formulation usually is preferred. However, with many proteins it is not possible—especially considering the time constraints for product development—to develop sufficiently stable aqueous formulations. Unacceptable denaturation and aggregation can be induced readily by the numerous stresses to which a protein in aqueous solution is sensitive; e.g., heating, agitation, freezing, pH changes, and exposure to interfaces or denaturants (Arakawa et al., 1993; Cleland et al., 1993; Brange, 2000; Bummer

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Samsung Bioepis Exhibit No. 1002
Page 8005

and Koppenol, 2000). Furthermore, even under conditions that thermodynamically greatly favor the native state of proteins, aggregation can arise during months of storage in aqueous solution (e.g., Gu et al., 1991; Arakawa et al., 1993; Chen et al., 1994; Volkin and Middaugh, 1996; Chang et al., 1996a). In addition, several chemical degradation pathways (e.g., hydrolysis and deamidation) are mediated by water. In aqueous formulations, the rates of these and other (e.g., oxidation) chemical degradation reactions can be unacceptably rapid on the time scale of storage (e.g., 18–24 months) for pharmaceutical products (Manning et al., 1989; Cleland et al., 1993; Goolcharran et al., 2000; Bummer and Koppenol 2000).

In contrast, a properly lyophilized formulation can maintain adequate physical and chemical stability of the protein during shipping and long-term storage even at ambient temperatures. As will be outlined in this chapter, developing stable lyophilized protein formulations should be a rational, straightforward process, which for most proteins should be rapid. With liquid formulation development, it may only be possible to obtain adequate protein stability after lengthy studies. Furthermore, sometimes there are conflicting conditions (e.g., pH) needed to slow sufficiently multiple degradation pathways in aqueous solution. Considering these issues plus the fact that formulation scientists now have to deal with numerous proteins and/or variants of a given protein, lyophilization should be considered as a primary mode for product development. Only if a parallel effort to develop an aqueous formulation is successful, will a final lyophilized product not be needed.

Rapid formulation development has important financial ramifications. A drug product has a finite patent life, during which time the company has an exclusive market. Considering that even a moderately successful drug product has annual sales of hundreds of millions of dollars, potentially millions of dollars in sales are lost for each day of delay in bringing a product to market. Unfortunately there are often delays because the formulations designed during early stage development and clinical trials (e.g., frozen) were not adequate for the final product. With a rational approach to formulation development, pharmaceutical scientists and process engineers can minimize the risk of this problem and the time needed to obtain a successful, final formulation. The success of such efforts depends on frank, open communication between the groups involved. For example, it is critical that the formulation scientists learn from the process engineers the issues for large-scale lyophilization runs, which are usually conducted in units that do not have the capacity to match processing parameters obtained in a small-scale research lyophilizers.

Despite the best efforts of the scientists and engineers, all too often delays in formulation development arise because sufficient resources are not invested in product development. For example, sometimes, purchase of essential equipmen

Samsung Bioepis Exhibit No. 1002
Page 8006

(e.g., a differential scanning calorimeter), which costs a minute fraction of a day's sale of product, is not allowed. To avoid unnecessary delays in product launch, which can have disastrous consequences for the company and for patients, it is essential that companies appreciate that product development is ultimately a key limiting factor in getting a therapeutic to market. Hence, development efforts need to be as well funded as the usually much more visible drug discovery research programs. If a product is not stable, it will not be marketed, no matter how dramatic an impact it can have on human health and the financial status of the company.

MINIMAL CRITERIA FOR A SUCCESSFUL LYOPHILIZED FORMULATION

Research over the past several years has demonstrated that five criteria define the minimal conditions necessary for a successful lyophilized protein formulation (Table 1).

The first four criteria can be met with use of the appropriate excipients and lyophilization cycle design. For information on the proper design of lyophilization cycles, the reader is directed to the numerous previous reviews in this area (Franks, 1990; Pikal, 1990; Nail and Gatlin, 1993; Gatlin and Nail, 1994; Carpenter and Chang, 1996; Rey and May, 1999; Cappola, 2000). For the current chapter we will only consider cycle design in terms of the interplay between formulation physical properties (e.g., collapse temperature) and process parameters (see below). The last criterion listed in Table 1 requires insight into the unique physicochemical properties of each therapeutic protein, which will be explained in more detail below. We will discuss in turn why each of these criteria is important. Then we will present an explanation of how to design rationally a formulation to meet these criteria.

Table 1.
Minimal Criteria for a Successful Lyophilized Protein Formulation

1. Protein unfolding during freezing and drying is inhibited.
2. The glass transition temperature of the product exceeds the planned storage temperature (e.g., $T_g > 30°C$).
3. The water content is relatively low (e.g., 1% by mass).
4. A strong, elegant cake structure is obtained (i.e., collapse and meltback are avoided).
5. Steps are taken to minimize specific routes of protein chemical degradation (e.g., product vials are sealed under nitrogen to reduce the rate of methionine oxidation).

Samsung Bioepis Exhibit No. 1002
Page 8007

Inhibition of Lyophilization-Induced Protein Unfolding

The stresses of freezing and drying cause protein unfolding, and the formulation must be designed to inhibit unfolding at each step (Prestrelski et al., 1993a,b; Carpenter et al., 1993; Prestrelski et al., 1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Chen et al., 1999; Bell, 1999; Carrasquillo et al., 2000). Even if the formulation excipients and/or intrinsic thermodynamic stability of the protein prevent denaturation during freezing, unfolding can arise during subsequent drying (Carpenter et al., 1993; Prestrelski et al., 1993b; Allison et al., 1998; Carrasquillo et al., 2000). Conversely, once a protein unfolds during freezing, it will not regain native structure during dehydration.

For many proteins, unfolding during lyophilization leads to clinically unacceptable, non-native aggregates, even when samples are rehydrated immediately after lyophilization (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a, 1999; Costantino et al., 1998). Aggregates are not necessarily formed during freezing and drying. Rather, during rehydration refolding of structurally perturbed protein molecules competes with formation of non-native protein aggregates (Prestrelski et al., 1993a). Aggregation can be minimized by including stabilizing excipients (e.g., sucrose or trehalose) in the formulation to inhibit lyophilization-induced unfolding (Prestrelski et al., 1993a, 1995; Allison et al., 1996; Krielgaard et al., 1998a, 1999; Costantino et al., 1998). Furthermore, fostering refolding during rehydration (e.g., with surfactants) can reduce aggregation (Chang et al., 1996c; Zhang et al., 1995, 1996).

In addition to minimizing protein aggregation during lyophilization/rehydration, maximizing retention of native protein structure in the dried solid is essential for optimizing long-term storage stability (Prestrelski et al., 1995; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Allison et al., 2000; Cleland et al., 2001). Both chemical and physical degradation in the dried solid can be accelerated if protein unfolding is not inhibited during lyophilization. With chemical degradation, a non-native structure may provide an environment conducive to covalent modification of one or more residues. For example, exposure of a methionine, which is normally buried deep in the interior of the native protein, on the surface of an unfolded dried protein may foster oxidation. Increased levels of aggregates noted after storage and rehydration of unfolded proteins could be due to formation of non-native intermolecular contacts within the dried solid, perturbation of refolding during rehydration because of chemical degradation, and/or other undefined processes.

Infrared spectroscopy has been used routinely to compare the secondary structures of a protein in lyophilized formulations to that of the native protein in aqueous solution (Prestrelski et al., 1993a,b; Prestrelski et al., 1995; Dong et al.,

Samsung Bioepis Exhibit No. 1002
Page 8008

1995; Constantino et al., 1995, 1998; Griebenow and Klibanov, 1995; Allison et al., 1996, 1998, 1999, 2000; Chang et al., 1996b; Krielgaard et al., 1998a, 1999; Carpenter et al., 1998; Chen et al., 1999; Carrasquillo et al., 2000). This method should be considered essential in the development of stable lyophilized formulations, because it allows one to assess rapidly the effectiveness of formulations at inhibiting protein unfolding. Technical details about how to employ infrared spectroscopy to design stable lyophilized protein formulations can be found in the papers cited above.

Storage at Temperatures Below Formulation Glass Transition Temperature

In the dried powder, the protein is a component of an amorphous phase that includes amorphous excipients and water. If this glassy matrix is held below its characteristic glass transition temperature (T_g), the rate of diffusion-controlled reactions, including protein unfolding/aggregation and chemical degradation, are greatly reduced, relative to rates noted at temperatures $>T_g$ (Roy et al., 1991; Franks, 1990; Franks et al., 1991; Pikal, 1994, 1999). T_g can be determined with differential scanning calorimetry (DSC) or other thermal scanning methods (Nail and Gatlin, 1993; Chang and Randall, 1992; Craig and Royall, 1998; Verdonck et al., 1999).

Obtaining a formulation T_g in excess of the planned storage temperature (e.g., room temperature) is absolutely essential for optimal protein stability (e.g., Franks et al., 1991; Pikal, 1994, 1999; Carpenter and Chang, 1996; Duddu and Dal Monte, 1997). The T_g of a given amorphous phase is dependent on the T_g and mass percent of each component, including water (Angell, 1995; Franks et al., 1991; Levine and Slade, 1988, 1992; Pikal, 1994, 1999). Compared to excipients, dried proteins have relatively high T_g's (e.g., >150°C; Angell, 1995). Thus, with all other factors being held constant, the formulation T_g varies directly with the mass fraction of protein. However, care must be taken that the mass fraction of protein is not so high that there are not adequate levels of stabilizing excipients to prevent protein unfolding during lyophilization (Cleland et al., 2001; and see below).

Fortunately, sucrose and trehalose, which are the preferred excipients for inhibiting lyophilization-induced protein unfolding (see below), also provide a glassy matrix with acceptably high T_g values. For example, with water contents of 1% the T_g for pure sucrose and trehalose are about 100 and 65°C, respectively (Crowe et al., 1998).

It has now been documented with several proteins, that simply storing the formulation at temperature below T_g alone does not assure optimal stability. A

Samsung Bioepis Exhibit No. 1002
Page 8009

native protein structure is also required. For example, proteins lyophilized in dextran alone are usually unfolded, but in a glassy matrix with a relatively high T_g (e.g., > 75° C). Yet they still degrade at relatively rapid rates compared to those for native protein molecules lyophilized with either sucrose or trehalose (Kriel-gaard et al., 1998a, 1999; Lueckel et al., 1998; Allison et al., 2000; Yoshioka et al., 2000). On pharmaceutical time scales of several months of storage many degradative reactions are not coupled to the glass transition of a formulation. This is because on these times scales there is still significant molecular mobility, even at temperatures well below (e.g., more than 30° C) the T_g (Hancock et al., 1995; Duddu et al., 1997; Pikal, 1999; Yoshioka et al., 1999).

The Water Content is Relatively Low

Because of its very low T_g (–135°C), water is a potent plasticizer for glasses; increasing water content in the dried formulation will greatly reduce T_g. For example, increasing the water content of pure sucrose from 1 to about 3–4% (g H_20/100 g dried powder) is sufficient to reduce the T_g to below room temperature (Crowe et al., 1998). It is critical to achieve a sufficiently low water level for a given formulation such that T_g exceeds the planned storage temperature. The lyophilization cycle dictates the initial water content (see reviews listed above). The most important parameter is the temperature for secondary drying, when the unfrozen water is desorbed (Pikal et al., 1990).

Water can also be transferred to the product from the vial stoppers during storage (Pikal and Shah, 1992; DeGrazio and Flynn, 1992; Hora and Wolfe, 1999). This effect can be dramatic. For example, let's consider a formulation containing 10 mg of dried protein, 40 mg of sucrose and initial water content of 1% by weight. The total amorphous fraction containing protein and sucrose has 0.50 mg of water. If 1.0 mg of water was transferred from the stopper to the product, the water content of this fraction would increase from 1% to 3.0%. This increase would be sufficient to lower the formulation T_g to below room temperature (Crowe et al., 1998). The risk of transfer of moisture from stoppers can be minimized by drying the stoppers before use, and, if acceptable for a given product, using stoppers coated with a material such as Teflon (see Hora and Wolfe, 1999).

A Strong, Elegant Cake Structure is Obtained

Often the most desired cake has strong, porous structure, without macroscopic collapse or meltback. This structure has a high surface area to volume

Samsung Bioepis Exhibit No. 1002
Page 8010

ratio, which aids in the rapid dissolution of product upon addition of water. A detailed account of how to obtain such a cake structure is beyond the scope of the current chapter, but is available in several previous reviews (see above). For the current purposes it is sufficient to focus on the impact of formulation composition on avoiding collapse or meltback. When a product is frozen, the protein and amorphous excipients (e.g., sucrose) are dispersed between ice crystals and any excipient used as a crystalline bulking agent (e.g., glycine). To obtain an appropriate cake structure during lyophilization, the product temperature during primary drying, when the water in ice is sublimed, must be below the characteristic collapse and eutectic melting temperatures of amorphous and crystalline solutes, respectively. Above the eutectic temperature, the melting of crystalline solutes leads to massive loss of porous structure and macroscopic dissolution of the frozen matrix into a "puddle". Above the collapse temperature, which closely coincides with the glass transition temperature (T_g') of the maximally freeze-concentrated amorphous phase, the amorphous phase cannot support its own weight. The result is also a loss of pore structure and a macroscopic shrinkage or collapse of the cake.

It should be noted that the T_g' thermal event, which can be measured with differential scanning calorimetry, is also referred to as a softening event (T_s), rather than the actual glass transition temperature of the freeze-concentrated amorphous phase (Shalaev and Franks, 1995). Whatever the exact nature of the thermal event, it can be detected with differential scanning calorimetry as a second order increase in the baseline of the thermogram, which usually occurs just prior to the onset of the endotherm for the melting of ice (e.g., Her and Nail, 1994). The transition can also be measured with electrothermal methods (Her et al., 1994).

Another powerful method, which is essential for rational development of lyophilized formulations, for determining collapse and eutectic melting temperatures is freeze-drying microscopy (e.g., Nail et al., 1994). With this approach, the formulation of interest is directly examined visually for its performance during a simulated freezing, annealing and drying cycle. All of the critical phase changes, including ice formation, solute crystallization, eutectic melting and collapse, can be detected easily, and the temperature of their occurrence can be measured accurately.

Formulation composition dictates collapse temperature. Each pure amorphous excipient has a characteristic T_g' and collapse temperature; the collapse temperature for the formulation is the mass averaged temperatures of all of the components in the amorphous phase. It is important to design a formulation with maximum collapse temperature, because the rate of drying is directly proportional to the sample temperature during lyophilization. To allow for a reasonable drying time the T_g' should not be lower than –40° C. The T_g' values for pure sucrose is –32° C, while that of pure trehalose is –30° C (Skrabanja et al., 1994; Chang and

Samsung Bioepis Exhibit No. 1002
Page 8011

Randall, 1992). In contrast, glucose, which should also be avoided because it is a reducing sugar, has a T_g' of −43° C. The collapse temperature of pure protein is about −10° C, which means increasing the protein:sugar mass ratio will increase collapse temperature. Finally, collapse temperature will be decreased if salts and excipients are not maximally crystallized. For example, glycine has a T_g' of −37° C, and its contribution to the amorphous phase can reduce collapse temperature to impractically low values (Carpenter and Chang, 1996). This problem can be avoided by using the appropriate annealing (see Carpenter and Chang, 1996) of the frozen product to maximize crystallization.

Meltback can be avoided by using crystalline solutes with relatively high eutectic melting temperatures. For example, eutectic melting temperatures for mannitol and glycine are −1 and −4° C, respectively. In contrast, additives such as calcium chloride have very low eutectic melting temperatures (e.g., −51° C for calcium chloride). If mannitol or glycine is used as a crystalline bulking agent, the T_g' of the amorphous excipient phase, which is lower that the eutectic melting temperatures of these excipients, will be the value that dictates the temperature of primary drying.

Collapse can also occur during secondary drying, when unfrozen water is desorbed, if the temperature is increased too rapidly. As water is removed from the amorphous phase the T_g of this phase increases. Thus, product temperature can be raised gradually and collapse can be avoided if product temperature at a given time point does not exceed the T_g. Directions for optimizing secondary drying can be found in previous reviews mentioned above.

Steps Taken to Minimize Specific Routes of Protein Chemical Degradation

It is essential that the major routes of chemical, as well as physical, degradation be characterized carefully for each protein, because all of the criteria listed above can be met and a protein might still be damaged during long term storage in the dried solid. For example, methionine oxidation is a common degradation pathway for therapeutic proteins (Manning et al., 1989; Ahern and Manning, 1992; Cleland et al., 1993; Goolcharran et al., 2000). Even in a formulation that prevents protein unfolding and has a T_g exceeding the storage temperature methionine oxidation can proceed at an unacceptably rapid rate. If a methionine residue is on the surface of the protein, maintaining native structure would not prevent this residue's exposure to reactive oxygen species in the dried solid. But why would the glassy matrix not sufficiently retard the mobility of the reactive species to prevent the reaction from occurring? On pharmaceutical time scales (i.e., of many months) relevant motion in a glass is not arrested unless the storage

Samsung Bioepis Exhibit No. 1002
Page 8012

temperature is about 50° C below the T_g (e.g., Hancock et al., 1995; Pikal, 1994; Pikal, 1999). Therefore, the mobility of a relatively small reactive oxygen species and the oxidation of methionine residues are probably not coupled to the glass transition of the formulation. An intriguing alternative, but not mutually exclusive, explanation has been suggested by Steve Prestrelski (personal communication). He proposes that reactive oxygen species accumulating during long-term storage might not be causing damage just in the dried solid. Rather upon rehydration oxygen radicals rapidly react with the protein. This is an important area for future research.

Fortunately, despite the rather complicated and poorly understood theoretical aspects of unacceptable methionine oxidation rates in dried protein formulations, from a practical viewpoint dealing with the problem is relatively straightforward. Methionine oxidation can be minimized by sealing vials under nitrogen and/or using formulation additives (e.g., free methionine) to compete with protein residues for reactive oxygen species. These approaches should be effective whether the oxidation of residues occurs in the dried solid, during rehydration or at both times.

RATIONAL DESIGN OF STABLE LYOPHILIZED FORMULATIONS

Two critical practical issues need to be considered when choosing excipients for a therapeutic protein formulation. 1) From a financial standpoint, scientists should focus on using excipients and processing approaches that do not need to be licensed from a patent holder. Acceptable protein stability can be achieved readily using excipients and processing methods that are well known to someone, to use the legal term, "skilled in the art of protein formulation." Clearly, however, the specific applications of such compounds and processes to a given protein drug product and/or a class of proteins often can be patented. Such patents may be critical to the company developing that product.

2) From a regulatory standpoint (and financial perspective, also), the formulation scientist should choose from among excipients that are already used in approved parenteral products. For protein stabilizers, the best choices are the disaccharides, sucrose and trehalose. For bulking agents, the best choices are glycine, mannitol and hydroxyethyl starch. For surfactants, usually the Tweens (20, 40 or 80) are preferred.

Based on these practical concerns and the criteria for a successful lyophilized protein product (Table 1), prototypic rational formulations are presented in Table 2. If the formulations suggested are employed, and the criteria in Table 1 are met, then most likely the product will have acceptable stability.

Samsung Bioepis Exhibit No. 1002
Page 8013

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Samsung Bioepis Exhibit No. 1002
Page 8014

Table 2.
Example of Rational Lyophilized Protein Formulation

1. Buffer that does not acidify during freezing (e.g., Tris, histidine, citrate)
2. Specific ligands/pH that optimizes thermodynamic stability of protein
3. Trehalose or sucrose to inhibit protein unfolding and provide glassy matrix
4. Bulking agent (e.g., mannitol, glycine or hydroxyethyl starch)
5. Nonionic surfactant to reduce protein aggregation

It is important to emphasize that not all components listed in Table 2 may be needed for a given protein product. For example, it may not be necessary to include a nonionic surfactant to aid in reducing protein aggregation. If there is not clear evidence that a given component is beneficial for a formulation, then that component should not be included in the formulation. Also, some components that are used because of "tradition" and/or for purposes such as final formulation tonicity can cause great difficulties in lyophilization. The most common example is the use of NaCl as a tonicity modifier. During lyophilization, NaCl can greatly reduce the collapse temperature of a formulation, if a fraction of the salt does not crystallize (Her et al., 1994). Crystallization of NaCl during freezing and annealing can be inhibited by other excipients (e.g., bulking agents and stabilizing sugars). Because of the low collapse temperature, a low temperature cycle must be used, which increases production time and costs. Also, even if formulations with NaCl can be lyophilized successfully in small research lyophilizers, there is great risk that a large fraction of vials will collapse during large scale manufacturing runs. Thus, if at all possible, NaCl should not be used in lyophilized formulations. Alternative tonicity modifiers include mannitol and glycine, which can also serve as crystalline bulking agents.

Every protein and product has unique characteristics, some of which may cause difficulty in designing stable formulations. Thus, the suggestions in Table 1 should be considered only as a good starting point in formulation development. For the remainder of this chapter we will discuss the rationale for the choice of each of the formulation components, their mechanisms of action and other practical approaches that can be used to increase protein stability.

Choice of Buffer

In terms of protein stability, the main concern with choice of buffer is the potential of certain buffer salts to precipitate during freezing and cause large changes in pH. For example, crystallization of the dibasic form of sodium

Samsung Bioepis Exhibit No. 1002
Page 8015

phosphate results can result in a pH < 4 (van den Burg, 1959; van den Burg and Rose, 1959; Anchordoquy and Carpenter, 1996). Thus, whenever possible, sodium phosphate buffer should be avoided. Although somewhat obvious, it is important to realize that a sodium phosphate system will be present if one starts with potassium phosphate buffer salts and NaCl, as is the case with phosphate buffered saline. Alternative buffers that do not appear to have major pH changes during freezing include Tris and histidine (Anchordoquy and Carpenter, 1996).

A clear example of the detrimental effects of buffer acidification on the stability of a lyophilized protein formulation has been reported by Lam et al. (1996). These researchers documented that a succinate buffer with an initial pH of 5 exhibited a pH drop of 1–2 units during lyophilization. The result was a reduced stability of interferon-γ during storage of the lyophilized formulation, relative to that noted for formulations prepared with glycolate buffer, which does not undergo freezing-induced acidification.

Specific Ligands/pH that Optimizes Thermodynamic Stability of Protein

Often experience gained during purification, intermediate storage protocols and preformulation studies can provide great insight into specific solution conditions (e.g., pH, ionic strength, buffer type) that confer the greatest stability to a given protein. Before choosing the appropriate "general" stabilizers, which are effective at protecting most proteins, it is absolutely essential that the formulation be optimized for the specific factors that increase the physical and chemical stability of a given protein. For example, simply avoiding extremes in pH can drastically reduce the rate of deamidation (Manning et al., 1989; Goolcharran et al., 2000). Also, specific ligands that increase protein physical stability (e.g., by increasing the free energy of unfolding) should be investigated. The stabilizing effects of heparin and other polyanions on growth factors (e.g., Chen et al., 1994; Volkin and Middaugh, 1996) and calcium on DNase (Chen et al., 1999) provide good examples. As will be described below, increasing the free energy of unfolding directly correlates with increased resistance of proteins to denaturation during freezing. In addition, at least in the case of DNase, there was also dramatic increases in storage stability of the lyophilized protein in formulation containing calcium, which were not due to increased structural stabilization during lyophilization (Chen et al., 1999). The mechanism for this effect is unknown.

Moreover, sometimes protein unfolding during freeze-drying can be minimized by optimizing initial solution pH (e.g., Prestrelski et al., 1995). However, in other cases it has been found that initial solution pH does not impact the degree of unfolding arising during lyophilization (Costantino et al., 1995; Carrasquillo et al., 2000). For each protein it is necessary to investigate the effect of initial pH

Samsung Bioepis Exhibit No. 1002
Page 8016

(and potential pH changes during freezing) on structural retention during lyophilization. Infrared spectroscopy can be used to monitor secondary structure in the dried solid, and the resulting data can be used to choose the initial pH that results in the most native-like secondary structure in the dried solid.

Trehalose or Sucrose to Inhibit Protein Unfolding and Provide Glassy Matrix

In this section we will discuss the rationale for choice of stabilizing excipients. Then we will describe the mechanisms for protection of proteins by additives during freezing and drying. Mechanistic insight is important for a clear understanding of protein stabilization during lyophilization and for guiding critical practical choices, such as determining the level of disaccharide needed for optimal protein protection. Finally, we will address this and other practical issues in the use of stabilizing excipients to inhibit protein unfolding during freezing and drying.

Rationale for Choice of Stabilizing Sugar. Among the numerous compounds tested, it appears that the most effective stabilizers of proteins during lyophilization are disaccharides (reviewed in Carpenter et al., 1999). Trehalose and sucrose are the best choices for a stabilizing disaccharide for therapeutic proteins. Both sugars: 1) protect proteins during both freezing and dehydration; 2) are nonreducing; 3) tend to remain amorphous during lyophilization; and 4) have been used in approved parenteral therapeutic products. There are, however, some important differences in the physicochemical properties of these sugars. 1) Trehalose has a higher T_g at a given moisture content than sucrose and, thus, for formulations containing trehalose it may be easier to obtain an appropriate cake structure with an economical lyophilization cycle (Crowe et al., 1998). However, a skilled process engineer should be able to design economical, effective cycles for formulations containing either sugar. In addition, the condition of having a T_g greater than the product storage temperature will hold at higher residual water contents for trehalose. In products with a relatively high protein concentration, the protein could contribute to an increased T_g, which serves to minimize the advantages of trehalose. 2) Trehalose is more resistant than sucrose to acid hydrolysis. Hydrolysis of these disaccharides produces reducing sugars, which must be avoided. Usually this is not a problem, unless very low pH's (ca. < 4) are employed. It should be noted that acid catalyzed hydrolysis of sucrose can occur even in lyophilized solids (Shalaev et al., 2000), suggesting that buffer acidification during freezing (see below) could ultimately result in formation of reducing sugars and resulting damage to proteins in a lyophilized formulation. 3)

Samsung Bioepis Exhibit No. 1002
Page 8017

Sucrose appears to be somewhat more effective at inhibiting unfolding during lyophilization (Allison et al., 1999). This difference has been most obvious when there is a relatively high protein concentration and a need to employ a relatively high initial concentration of sugar. Evidence to date indicates that less effective stabilization by trehalose is due to the greater propensity of this sugar to phase separate from polymers (Isutzu et al., 1996) and proteins (S.D. Allison, T.W. Randolph, B.S. Chang and J.F. Carpenter, unpublished observation) during freezing and drying. Whether or not this is a problem with a given formulation cannot be predicted. Hence, the relative capacities of sucrose versus trehalose to inhibit lyophilization-induced unfolding of must be examined for each protein. The final choice of sugar for a given protein product should be based on a direct comparison of sucrose and trehalose as stabilizers during the lyophilization cycle and storage in the dried solid.

Reducing sugars, such as maltose or lactose, should not be used. These compounds may effectively inhibit protein unfolding during the lyophilization cycle, but during storage in the dried solid they can degrade proteins via the Maillard reaction between carbonyls of the sugar and free amino groups on the protein (Hageman, 1992; Li et al., 1996).

Mechanism for Freezing Protection. Many different compounds, such as sugars, polyols, certain amino acids, methylamines and salting-out salts, nonspecifically stabilize proteins during freezing or freeze-thawing ("cryoprotection"). The same compounds have also been shown in nonfrozen aqueous systems to increase protein thermodynamic stability (e.g., increase resistance to temperature- or chaotrope-induced unfolding). Protection of proteins by these compounds during freezing and freeze-thawing is due to the same universal thermodynamic mechanism that Timasheff and colleagues have defined for solute-induced protein stabilization in nonfrozen aqueous solution (reviewed in Carpenter and Crowe, 1988; Carpenter and Chang, 1996; Carpenter et al., 1999; Timasheff, 1998).

Usually relatively high concentrations (ca. > 0.3 M) of solute are needed to stabilize proteins because the interactions of the solute with the protein are relatively weak. Stabilizing solutes are excluded preferentially from the surface of the protein. This statement does not mean that no solute molecules bind to the surface of the protein, but rather that there is a lower concentration of solute in the immediate vicinity of the protein than in the bulk solution. The chemical potential of the protein is increased in the presence of a preferentially excluded solute, which is the basis for the effect of solute of protein thermodynamic stability. Considering a two-state model for protein folding (N $\leftarrow \rightarrow$ D), the native state is favored thermodynamically (e.g., under physiological conditions) because it has a lower free energy than the denatured state. The key to increasing the thermodynamic stability of the native state is to increase the free energy barrier

Samsung Bioepis Exhibit No. 1002
Page 8018

between the native and denatured states. So how can increasing the chemical potential of the native state result in stabilization? The degree of preferential exclusion and concomitant increase in protein chemical potential correlates directly with the protein surface area. The denatured state has a much greater surface area than the native state. Thus, in the presence of a preferentially excluded solute, the magnitude for the increase in protein chemical potential will be much greater for the denatured than that for the native state; the native state will be stabilized.

This same mechanism applies to the inhibition of smaller scale structural expansions of the native state, which may be sufficient to promote irreversible protein aggregation, by compounds such as sucrose (e.g., Chang et al., 1996a; Kendrick et al., 1997, 1998; Kim et al., 2000). Many proteins are known to form non-native aggregates from species with conformations that are not greatly different from the most compact native state. One example is the molten globule that has perturbed tertiary structure, but native secondary structure. However, even expanded species within that native state ensemble can participate in intermolecular interactions leading to non-native protein aggregates (e.g., Chang et al., 1996a; Kendrick et al., 1997, 1998; Kim et al., 2000). Preferentially excluded solutes shift the equilibrium between protein species towards that with the lowest surface area, i.e., the most compact species in the native state ensemble. As a result aggregation is inhibited.

Timasheff's preferential interaction mechanism also explains the influence of solutes on the degree of assembly of multimeric proteins. Preferentially excluded solutes tend to induce polymerization and stabilize native oligomers since the formation of contact sites between constituent monomers serves to reduce the surface area of the protein exposed to the solvent. Polymerization reduces the thermodynamically unfavorable effect of preferential solute exclusion. In this case, the assembled protein is much more stable than the constituent monomer because of the direct solute effects, but also because of the increase in oligomer stability gained by intersubunit contacts (e.g., Neet and Timm, 1994). In one example of this effect during lyophilization, maintenance of the native tetramer during freezing has been shown to increase the resistance of lactate dehydrogenase to dissociation and inactivation during subsequent drying (Anchordoquy and Carpenter, 1996; Anchordoquy et al., 2001).

How do we know that the preferential exclusion mechanism is actually operative in the frozen state? It is not possible to measure directly the protein-solute interaction in the frozen state. However, the effects of solutes on protein chemical potential and the resulting protein stabilization can be inferred from a freezing study with hemoglobin, in which the protein was partitioned into separate polyethylene glycol-rich and dextran-rich solution phases (Heller et al., 1996). In a phase separated system, thermodynamic equilibrium requires that chemical potential of each component be equal in both phases. Hence, there should be

Samsung Bioepis Exhibit No. 1002
Page 8019

equivalent effects of the different solute conditions in the two phases on the increase in free energy of protein unfolding. In other words, the impact of preferentially excluded solutes on protein stability is equal in the two phases. As predicted from the preferential exclusion mechanism, the degree of structural protection of hemoglobin during freezing (as observed directly in the frozen state with infrared spectroscopy) was equivalent in both phases (Heller et al., 1996).

Mechanism for Inhibition of Dehydration-Induced Unfolding. The interaction of a protein's residues with water are intimately involved with the formation of the native, globular protein structure, and if the protein is dehydrated it will unfold. It has been documented by numerous studies that sucrose and trehalose prevent dehydration-induced unfolding by hydrogen bonding to the dried protein in place of the lost water (e.g., Carpenter and Crowe, 1989; Prestrelski et al., 1993a; Allison et al., 1999; Costantino et al., 1998; Wolkers et al., 1998; Tzannis and Prestrelski, 1999). This so called "water replacement mechanism" is supported by several different observations.

For example, with infrared spectroscopy, it has been found that the band at 1583 cm^{-1} in the spectrum for lysozyme, which is due to hydrogen bonding of water to carboxylate groups, is not present in the spectrum for the dried protein (Carpenter and Crowe, 1989; Remmele et al., 1997; Allison et al., 1999). When lysozyme is dried in the presence of trehalose or sucrose, the carboxylate band is retained in the dried sample, indicating that the sugar is hydrogen bonding in the place of water. Similar results have been obtained with α-lactalbumin and sucrose (Prestrelski et al., 1993a). The magnitude of the retention of the carboxylate band correlates directly with the level of trehalose or sucrose in the lyophilized formulation, as well as with the degree of inhibition of unfolding (Allison et al., 1999). These effects of sugars on proteins in the dried solid are not due to the presence of increased amounts of water in the formulations dried with sugars (Allison et al., 1999; Tzannis and Prestrelski, 1999). The level of water in formulations dried with the sugars is as low as that for the protein lyophilized from just buffer or water, and is so low that the hydration shell of the protein is essentially completely removed (Prestrelski et al., 1993a; Krielgaard et al., 1998a, 1999; Allison et al., 1999).

Furthermore, Farhat et al., (1998) used infrared spectroscopy to study dried gelatin-sugar mixtures and the hydration behaviors of the mixtures, and concluded that the sugars hydrogen bond to the dried protein. Costantino et al. (1997) studied water sorption of proteins lyophilized with sucrose or trehalose and found that the solid-state interactions between protein and sugar reduced the availability of water binding sites. Tzannis and Prestrelski (1999) found that water sorption behavior of spray-dried protein formulations containing stabilizing levels of sucrose indicated hydrogen bonding between dried protein and the sugar. However, if an excessively high sucrose:protein ratio was employed, due to

Samsung Bioepis Exhibit No. 1002
Page 8020

formation of protein- and sugar-rich phases, hydrogen bonding of sucrose to protein and resulting protein stabilization were reduced. Also, Suzuki et al. (1998; 1999) found that there was a high degree of stabilization of lactate dehydrogenase when sucrose remained amorphous and hydrogen bonded to the dried protein. Crystallization of sucrose abolished hydrogen bonding between the sugar and dried protein, as well as protein stabilization.

Further support for the water replacement mechanisms comes from two studies that compared the relative effectiveness of saccharides of increasing molecular weight for inhibiting lyophilization-induced unfolding. Tanaka and colleagues (1991) found that the capacity to protect catalase during freeze-drying was inversely related to saccharide size. They suggested that as the size of the saccharide increases, steric hindrance interferes with hydrogen bonding between the saccharide and the dried protein. In support of this contention, the carboxylate band is only minimally detectable in the infrared spectrum of lysozyme freeze-dried in the presence of glassy dextran and the protein is unfolded (Allison et al., 1999). With infrared spectroscopy, Prestrelski et al. (1995) found that as the molecular weight of a carbohydrate additive was increased the capacity to inhibit unfolding of interleukin-2 during lyophilization decreased, and the level of protein aggregation after rehydration increased. Also, it was clear that protection of the protein did not correlate directly with the formation of a glass (all samples were found to be amorphous) or with the glass transition temperature of the sample (the T_g increased as carbohydrate molecular weight increased). Rather, there was a negative correlation between stabilization and molecular weight, which is to be expected if protection during drying is due to the water replacement mechanism.

An alternative mechanism for stabilization of proteins during dehydration states that proteins are simply mechanically immobilized in a glassy, solid matrix during dehydration (e.g., Franks, 1991). The restriction of translational and relaxation processes is thought to inhibit protein unfolding, and spatial separation between protein molecules (i.e., "dilution" of protein molecules within the glassy matrix) is proposed to prevent aggregation. It is clear that protective additives must partition with the protein into the amorphous phase of the dried sample. If the compound crystallizes during lyophilization it does not inhibit protein unfolding (Carpenter et al., 1993; Izutsu et al., 1993; Krielgaard et al., 1999). Also, spatially separating protein molecules can help favor refolding over aggregation during rehydration (Allison et al., 1998, 2000). However, as evidenced by the failure of glassy dextran matrices to prevent unfolding (Prestrelski et al., 1995; Krielgaard et al., 1999; Allison et al., 1998, 1999, 2000), simply forming a glassy solid is not sufficient for inhibiting dehydration-induced unfolding.

Practical Considerations. The protein mass in a unit dose (or multidose) vial of therapeutic protein is dictated by the amount to be given to the patient.

Samsung Bioepis Exhibit No. 1002
Page 8021

With a lyophilized product, which can be reconstituted to a volume different from the prelyophilization volume, the initial protein concentration can be varied without changing the total protein mass. Changes in initial protein concentration can affect relative loss of protein due to interaction with vial surfaces (e.g., Page et al., 2000), protein stability during processing, the collapse temperature of the formulation, the T_g of the final dried formulation and the lyophilization process itself. Thus, protein concentration can be an important variable to investigate during formulation development.

Increasing initial protein concentration leads to apparent increased resistance to denaturation during freezing (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). This phenomenon can be demonstrated by determining the percentage protein aggregated after freeze-thawing, which varies inversely with protein concentration. Increasing protein concentration can directly reduce freezing-induced protein unfolding because one component of protein damage during freezing appears to involve protein denaturation during formation of the ice-water interface (Strambini and Gabellieri, 1996; Chang et al., 1996c; Krielgaard et al., 1998b). Assuming that only a finite number of protein molecules can be unfolded per unit area at this interface (Krielgaard et al., 1998b), increasing the initial protein concentration will lead to a smaller percentage of damaged molecules. Of course, other factors such as direct cold denaturation and freeze concentration of salts may predominate over surface denaturation during freezing. For practical purposes, it is not necessary to sort out the relative contribution of the various stresses to freezing-induced damage. Rather, is important to include realative high protein concentrations during early formulation development in order to increase the "intrinsic" resistance of the protein to denaturation. The greater the intrinsic stability of the protein, the lower the concentration of excipient that will be needed.

Another advantage of employing a relatively high initial protein concentration is that increasing protein : disaccharide mass ratio increases both formulation collapse temperature and T_g' of the final dried product. In addition, the formulation volume will be reduced, which will reduce the duration and costs of lyophilization.

Of course, if the ratio of excipient to protein is not sufficiently high, there will not be adequate stabilization of the protein during freezing and dehydration. Freezing protection depends on the initial bulk concentration of the sugar, and if the given protein is freeze-labile sometimes concentrations exceeding 5% (wt/vol) are needed to maximize stabilization. However, often the stability of the protein is sufficient during freezing that the level of sugar needed for optimal protection during lyophilization is dictated by that required for inhibition of unfolding during dehydration. Protection during drying depends on the final mass ratio between the sugar and the protein (Pikal, 1994; Carpenter and Chang, 1996;

Samsung Bioepis Exhibit No. 1002
Page 8022

Cleland et al., 2001). Recently it has been demonstrated that the sugar:protein mass ratio predicts not only degree of native structural retention during lyophilization, but also the long-term storage stability of a lyophilized protein (Cleland et al., 2001). Generally, a weight ratio of sugar to protein of at least 1:1 is required for inhibiting lyophilization-induced unfolding, with optimal stability being reached at around 3–5:1. In practice, with the protein concentration held constant, a range of sugar concentrations can be tested during formulation screening to discern the optimal concentration needed for retention of native protein in the dried solid. In general, the optimal sugar concentration for stabilizing the protein during lyophilization will also provide storage stability, if the final dried powder has a T_g well above the storage temperature. Finally, it should be stressed that the minimal effective level of sugar should be employed, because of the effect of changing protein:excipient ratio on glass transition temperatures. Also, if excess levels of sugar are employed there is an increased risk of sugar crystallization if a product should happen to be held at temperatures near or above the formulation T_g (e.g., Krielgaard et al., 1999).

Bulking Agent (e.g., Mannitol, Glycine or Hydroxyethyl Starch)

If the product has a relatively low mass of protein per vial, often it will necessary to have a bulking agent in the formulation to prevent the protein from being lost from the vial during drying and to form the product cake. Mannitol and glycine are examples of bulking agents, which can also serve as tonicity modifiers, that usually crystallize to a substantial degree during lyophilization (Pikal, 1994; Carpenter and Chang, 1996). A disaccharide protein stabilizer will need to be used in combination with these bulking agents. One drawback of mannitol and glycine is that often an annealing step is required to assure maximum crystallization. If a fraction of the bulking agent remains amorphous during lyophilization, there may be problems with obtaining a suitable cake structure, as well as a risk of excipient crystallization during subsequent storage in the dried solid (e.g., Carpenter and Chang, 1996; Carpenter et al., 1997). An alternative, amophous bulking agent is the polymer hydroxyethyl starch, which is used clinically as a plasma volume expander. Its main advantages in lyophilization are that it has a high collapse temperature (ca. −10°C), it forms strong cakes and it has a relatively high T_g for a given moisture content (e.g., >200°C at 2% residual moisture). However, as is the case with dextran, the large polymer hydroxyethyl starch does not inhibit protein unfolding during dehydration, and may actually foster additional protein unfolding due to phase separation from the protein during freezing and drying. Again, stabilizing disaccharides must be used with hydroxyethyl starch to inhibit lyophilization-induced protein unfolding.

Samsung Bioepis Exhibit No. 1002
Page 8023

Nonionic Surfactant to Inhibit Aggregation

Surfactants are often included in protein formulations to minimize interfacial denaturation and aggregation, e.g., at vial surfaces, due to bubble entrainment during filling or because of agitation (Chang et al., 1996c; Krielgaard et al., 1998b; Page et al., 2000). For a lyophilized product, a surfactant can be beneficial to minimize protein aggregation during vial filling, freezing and rehydration. Generally, a surfactant will not inhibit protein unfolding during dehydration (Krielgaard et al., 1998a). The mechanisms by which surfactants inhibit protein aggregation will be described in a separate chapter in this volume. For the current discussion it is sufficient to stress that a surfactant should not be included in a lyophilized product, unless there is direct evidence that increases recovery of native protein in the rehydrated sample. Surfactants can decrease the free energy of unfolding of some proteins, which may cause the compound to actually foster aggregation. Also, they have been shown to inhibit the assembly of small soluble aggregates into higher order soluble oligomers and insoluble aggregates (e.g., Krielgaard et al., 1998b). This effect can manifest itself during lyophilization and rehydration. As a result, the presence of a surfactant can cause an undesirable increase in the level of soluble aggregates.

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Samsung Bioepis Exhibit No. 1002
Page 8029

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Samsung Bioepis Exhibit No. 1002
Page 8030

8

High Throughput Formulation: Strategies for Rapid Development of Stable Protein Products

Rajiv Nayar and Mark C. Manning

INTRODUCTION

With the growing demand for new and innovative medicines, drug companies are spending record amounts of money on research and development. In the U.S. alone, research and development (R&D) investments are expected to exceed $24 billion in 1999, with investments in biotechnology totaling about $7 billion (Pharmaceutical Research and Manufacturers of America, 1999). As a result, new technologies are increasing the efficiency of the drug discovery process, and the drug pipelines have more products than ever in development. Nearly 350 biotechnology-related products are currently in clinical trials and over 50 are on the market (Pharmaceutical Research and Manufacturers of America, 1999). Pharmaceutical companies can now typically assess potential activities of up to 100,000 compounds a day using high throughput screening systems. In the biotechnology arena, recent advances in genomics, functional genomics, proteomics, bioinformatics and pharmacogenomics are facilitating the development of protein drug candidates at a much faster rate than was possible during the early years of the biotechnology industry. Couple these changes with the impending

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Samsung Bioepis Exhibit No. 1002
Page 8031

publication of the complete sequence of the human genome by 2001 and there is a potential for an additional 15,000 protein drugs from the predicted 150,000 potential genes in the human genome.

This new wave of protein drugs will include compounds that can function as growth factors, act as specific stimulators or suppressors of certain functions or exhibit activities that have not been observed before. Many experts anticipate a golden age of protein-based therapeutics in the twenty-first century. As various research enterprises gear up to search for these new candidates using high throughput cellular and molecular screening assays, there undoubtedly will be a need for a corresponding ability to formulate these drugs much more quickly than in the past. In addition, there will be increased use of novel delivery systems to administer these protein therapeutics efficiently, making stabilization even more challenging. In short, the pharmaceutical biotechnology industry must find new ways to speed up the drug development process, in order to keep pace with the drug discovery process. We think that the limiting factor for introduction of new biotechnology-derived products will not be finding new drug candidates, but efficient and rapid formulation of these compounds into acceptable dosage forms for delivery and testing in humans.

The potential of many new drug candidates flowing through the pipeline will require implementation of an entirely new approach towards product development. The particular challenge addressed here is the demand that will be placed upon formulation groups, where there will be increased expectations to develop stable formulations of:

more proteins,
in a shorter period of time,
with less material, and
with little, if any, increase in personnel.

Therefore, one must consider new strategies for meeting such challenges.

This chapter is intended to serve as a forum for discussing and evaluating what factors must be considered in trying to implement new approaches to formulating protein pharmaceuticals in a rapid, rational fashion. As a comprehensive strategy for developing stable formulations of protein pharmaceuticals is not currently available, we have assembled what we consider to be the key elements necessary to accomplish this critical task. This chapter should not be viewed as a finished product, but as a work-in-progress, encouraging pharmaceutical scientists to comment on the concept and to modify the approach to their particular situations.

Again, the overarching goal is to possess a unified strategy for accommodating many more proteins at one time, while meeting shortening timelines. It should be expected that there would be a limited supply of well-characterized material and a fixed personnel head count. We envision a scheme that we term "high throughput formulation" or HTF. It is based upon our current

Samsung Bioepis Exhibit No. 1002
Page 8032

understanding regarding stabilization of proteins, but it also identifies areas where technical improvements must be made, especially in the area of analytical methodology and software. If the HTF strategy is successful, it should be possible to match the output of stable formulations by development groups with potential leads by discovery groups.

OVERALL STRUCTURE OF THE HTF APPROACH

The HTF concept includes four key elements, which are briefly outlined in Table 1. The heart of the HTF strategy is a decision plan, where the formulation scientist must decide on (a) the specific excipients to be used as well as (b) the final dosage form. Central to the HTF scheme is the use of a limited set of additives as well as dosage forms. Especially for lyophilized or freeze-dried formulations, our understanding of the behavior is such that some standard formulations can be envisioned (see Chapter 5). Also, the choice of basic solution conditions will be based upon preformulation studies that examine the behavior of the protein as a function of pH, ionic strength, and buffer type (see Chapters 1 and 3).

The second key aspect is an increased role for software and information databases. The preformulation studies could require a significant number of independent experiments to be conducted. Therefore, experimental design software should be used to reduce the number of experimental trials and optimize the results through statistical evaluation. In addition, there are a number of databases and associated programs for assessing physical and chemical properties based upon primary sequence of the protein through a number of websites (e.g. SWISS-PROT). These can provide insight into protein structure and other critical characteristics (e.g., pI, hydrophobicity and hydroflexibility plots and overall charge), and even identify residues that might be sensitive to chemical decomposition. We believe such capabilities will expand over time, and will play an increasingly important role in setting a rational plan for preformulation studies. Finally, we anticipate that specific protein stability databases will emerge, allowing scientists to gather data on specific development issues, without searching all of the open literature. Because information on protein stability comes from a multitude of disciplines (e.g., biochemistry, biophysics, food science, engineering, pharmaceutics, surface science and polymer science), open searches often miss crucial publications and documents. The challenge is not unlike Internet search engines trying to find a very specific subset of information on an ever-increasing world wide web. As these resources become more available and refined, they will emerge as invaluable tools for development of pharmaceutically acceptable formulations.

Samsung Bioepis Exhibit No. 1002
Page 8033

Table 1.
Key Elements of the HTF Approach

1. Decision Plan to Finalize Choice of Excipients and Dosage Form
   - A. Choice of Dosage Form
     1. Frozen Solution
     2. Liquid Formulation
     3. Lyophilized Formulation
     4. Protein Suspension
     5. Specialized Drug Delivery System
   - B. Choice of Excipients
     1. Proper pH/Buffer Species
     2. Salt
     3. Stabilizer
     4. Bulking Agent (for lyophilized formulations)
     5. Surfactant
     6. Chelators
2. Use of Software and Databases to Assist in Formulation Development
   - A. Experimental Design Software
   - B. Software and Databases for Analysis of Structure/Function/Properties/Stability
   - C. Protein Stability Databases
3. Availability of Essential Analytical Methods
4. Design of Appropriate Stability Testing Protocols

The third aspect of HTF is assembling the correct analytical methods to assay the stability, both chemical and physical, of a wide range of proteins. The mechanisms by which proteins degrade have been widely reviewed (Manning et al., 1989; Pearlman and Nguyen, 1992; Cleland et al., 1993), so they will not be discussed here. However, given this backdrop, we will describe certain key methods that must be available in order to conduct proper HTF studies. All of the required instruments do not necessarily need to be available in-house, as many can be accessed through contract organizations and through academic collaborations. In addition, it should be noted that there exists a tremendous opportunity to modify existing analytical techniques for use with smaller sample sizes and for rapid, parallel sampling. One of the expected limitations will be lack of well-characterized material. Whereas access to a minimum of 200–500 mg of protein for formulation studies is desirable, the genomic approach will tax the ability of process scientists to provide adequate amounts of purified proteins for all compounds under development. Therefore, in some cases, the total amount of protein available may be an order of magnitude less than the optimum. This situation should serve as an incentive to identify new approaches for gathering stability data. We will discuss below possible new methodologies that would be useful in the HTF scheme, but have not yet been applied to formulation development.

Samsung Bioepis Exhibit No. 1002
Page 8034

Finally, one must have a clear plan for stability testing, along with minimum criteria for a "stable" formulation. Certainly, that definition may change as a product matures. Minimum stability for the initial clinical studies may be quite different than the expectations for the final formulation tested in Phase III. Presently, there are debates within companies on how robust a preliminary formulation development or purification process must be in order to demonstrate efficacy in humans. Some companies will accept any minimal formulation in order to initiate clinical trials. However, even these formulations would likely have to have at least six to twelve months of shelf life. Our goal in the HTF approach is to develop a final formulation as early as possible in the drug development and testing process. If this goal can be accomplished, then fewer resources will need to be diverted to redesign the formulation later.

ROLE OF AN ESTABLISHED DECISION TREE FOR FORMULATION DESIGN

The central feature in the HTF scheme is a rational decision plan for choosing both the formulation components and the final dosage form. This decision tree must be flexible enough to accommodate a wide variety of proteins, it must allow for both liquid and solid dosage forms, and it must provide a rationale at each decision point. This framework allows the pharmaceutical scientist to decide what types of formulations are worth considering, both in terms of stability and the demands and desires of the marketing and clinical groups.

Often the design of a formulation of a protein pharmaceutical is not well developed. Companies attempt to expedite formulation selection by neglecting critical issues in formulation and stability. It is not uncommon to find sub-optimal formulations such as phosphate-buffered saline, possibly with the addition of human serum albumin as a ubiquitous protein stabilizer. For a few proteins, this approach has been sufficient, even resulting in commercially successful products, such as erythropoietin and granulocyte colony stimulating factor. However, there is a distinct danger in assuming that such successes with minimal formulations are generally applicable. Moreover, the current regulatory environment, and concerns in the general public surrounding the use of human-derived products in pharmaceuticals, makes the use of human serum albumin increasingly more difficult or even not acceptable. Therefore, there is pressure for any company selling a product that contains albumin to develop an albumin-free formulation. The same pressures are forcing companies to move from animal-derived surfactants to those obtained from vegetable sources. In addition, there is a realization that albumin, being a protein, is not indefinitely stable, and should have an expiration date above and beyond that of the active ingredient. All of these issues affect the

Samsung Bioepis Exhibit No. 1002
Page 8035

list of possible excipients that can be employed in a protein pharmaceutical product.

Constraints on a Pharmaceutically Acceptable Protein Formulation

Before we discuss the choice of dosage forms and excipients in the context of maximizing protein stability, we need to recognize the myriad of constraints placed upon the formulation by the rest of the company and by regulatory bodies. The first constraint, given the global nature of the pharmaceutical industry, is to have excipients that are acceptable in the major markets. This includes avoiding animal-derived additives, as discussed above. It also means that the list of possible additives is effectively limited to those already found in approved products in North America, Europe, and Japan. While this is not an immutable rule, few companies are willing to bear the added cost of getting a new excipient on the market while seeking approval for a new drug product. A major impact of this constraint is to move away from formulations containing albumin, which is still found in a number of protein products. One can anticipate that these older formulations will disappear in the coming years and be reformulated into second-generation products. A recent example has been the development of an albumin-free formulations for recombinant Factor VIII.

The second consideration is the tonicity of the final formulation. If a protein drug is to be administered by intravenous bolus injection or subcutaneously, rather than by continuous infusion, there are strict isotonicity and pH considerations that have to be met for a pain-free injection. Similarly, there are constraints on the choice of excipients, as some (e.g., citrate) have been shown to be irritating upon injection. This is one example of restrictions being set by the clinical groups, which should be clearly defined prior to the start of the formulation process.

Likewise, the eventual route of administration must be taken into account. A convenient administration system is highly desirable for ease of use in the clinical setting, so it is both a marketing and clinical issue. For example, in the case of a subcutaneous injection, there is a maximal volume (~1 ml.) that can be given to a patient without discomfort. Given the expected dose range, this will define the protein concentration that must be used. For other routes of administration, the ability to manipulate the protein concentration as a variable may be possible. Otherwise, it will be fixed, and the decision point regarding protein concentration is removed from the process described below.

The fourth issue is to work with a well-characterized bulk drug substance. This criterion will require cooperation and collaboration with the process science groups involved in fermentation and purification. Variation in the quality of the

Samsung Bioepis Exhibit No. 1002
Page 8036

starting material will compromise the validity of any preformulation or stability work done to date, as well as potentially invalidate data from toxicological tests.

The fifth constraint to be determined is the minimal stability required to complete proof-of-principle investigations, meaning animal studies and initial clinical trials. These investigations can extend for up to one year. Hence, at least a one-year shelf life for even the initial formulation may be required.

Finally, the manufacturing of the protein product must be possible at reasonable costs and allow adaptation to standard manufacturing processes. It is imperative that manufacturing economic analysis be conducted as soon as possible in the drug development process, including an assessment of whether sufficient manufacturing capability exists if the product is successful. Novel formulations that require special manufacturing setups and processes may not offer the advantages of speed which is necessary when one is confronted with development of numerous protein drug candidates. Most likely, specialized dosage forms and/or drug delivery systems will only be investigated with partners who possess the technology and capacity to solve these critical manufacturing issues. In addition, as the in-house pilot plants are challenged with manufacturing multiple products, with limited facilities and resources, there undoubtedly will be pressures to outsource the first clinical manufacturing because of both cost-effectiveness and timescale issues.

Proper Choice of Dosage Form

Considering all the therapeutic protein products on the market, it appears that three dosage forms are viable. The most preferred would be a solution formulation that is typically stored in the refrigerator and preferably in a pre-filled syringe. Second, a frozen solution loaded in a syringe or vial and thawed at the site of administration. Third, a lyophilized formulation, which can be stored at room temperature and reconstituted when needed. Each one of these dosage forms offers various advantages and disadvantages, in the speed of development, manufacturing, packaging and shipment logistics, and in administration of the product to the patient.

Generally, a frozen protein formulation offers the advantages of rapid development provided the protein is stable after multiple freeze/thaw cycles. It is also important to note that freezing can lead to selective precipitation of certain buffer species, producing possibly large pH shifts (van den Berg, 1959; van den Berg and Rose, 1959). This variation in pH, and the potential for damage at the water-ice interface (Chang et al., 1996; Hsu et al., 1995; Krielgaard et al., 1998), might limit the use of such a dosage form. Furthermore, there are disadvantages associated

Samsung Bioepis Exhibit No. 1002
Page 8037

with a frozen formulation concerning the packaging complexities involved in labeling, storage and maintaining the cold chain during transportation of a frozen product. These factors can add significantly to the development costs.

The second option for dosage form is a stable liquid formulation that can be stored in the refrigerator or at room temperature. This dosage form offers the advantages of potentially rapid development and ease of administration. However, for most proteins maintaining physical and chemical stabilities in aqueous solution for an extended period of time is extremely difficult. In addition, because the protein is free in solution there is potential for adsorption to the vial, whether it is made of glass or some type of plastic. A liquid product will have to be shown to be impervious to damage at the air-water interface during transportation, because agitation-induced denaturation and aggregation is a common problem with therapeutic proteins. There is also the possibility of leaching of metals and organics from the container and stopper, which is why some of the vials will need to be inverted during stability testing. These problems have been reported for drug products of all types (Airaudo et al., 1990; Nedich, 1983). Leached materials are particularly problematic with plastic vials and non-Teflon-coated stoppers. With glass vials the leachables could include ionic species that alter the pH and ionic strength of the solution during storage. Finally, the integrity of the container closure must be demonstrated, in order to ensure sterility of the product. For multi-use vials, preservatives can be added, but some are known to foster degradation of proteins (Lam et al., 1997; Rodrigues-Silva et al., 1999). Therefore, development of a stable liquid protein formulation has to be considered very carefully. Determination of the primary pathways responsible for decomposition of the active therapeutic protein is critical for developing this particular dosage form. All of these issues can add significant costs in terms of resources and analytical testing.

Most protein pharmaceuticals currently on the market are sold as lyophilized formulations. Placing proteins in the dried state significantly increases the shelf life, if the proper formulation and lyophilization cycle are used. The combined stresses of drying and freezing are inherently damaging to proteins, and only proper formulation will ensure that a stable product will be obtained. Fortunately, enough is understood now about freezing and drying of proteins that rational design of lyophilized formulations can be undertaken. Moreover, there are some formulations that seem to work well for a variety of proteins. We suggest that part of the strategy for rapidly identifying viable formulations is to make liberal use of some standard protein formulations. The rationale for these compositions has been described in detail in a number of review articles and book chapters, and is discussed in Chapter 5 (MacKenzie, 1976; Franks, 1990; Pikal, 1985, 1994; Carpenter and Chang, 1996; Carpenter et al., 1997). An approach towards developing a lyophilized formulation is described below.

Samsung Bioepis Exhibit No. 1002
Page 8038

As other formulation possibilities become more developed, one can modify the decision tree to accommodate them. For example, one can imagine alternatives to lyophilization for obtaining solid dosage forms. Both spray-drying and spray-freeze drying are being studied. However, it is expected that the formulation strategy for these processes will be quite similar to that used for lyophilization.

The most likely future additions to the dosage form list will be suspensions and specialized drug delivery systems. Protein suspensions can be produced by a number of different methods. The protein could be dried and suspended in a non-aqueous medium. Alternatively, the protein could be salted-out, using preferentially excluded solutes, such as ammonium sulfate or sucrose, leading to an aqueous vehicle containing most of the protein in its native state but in a solid form, because the solubility limit has been exceeded. The same effect might be accomplished by adjusting the pH to be near the isoelectric point of the protein. For the current version of HTF, we have chosen not to include these dosage forms until their use becomes more widespread and manufacturing facilities are developed to accommodate production of clinical batches.

The same can be said of specialized drug delivery systems, such as polymeric microspheres. Poly lactide/poly glycolide microspheres for sustained delivery of peptides have been in use for years. Recently, reports of their use for proteins, such as human growth hormone, have appeared. These systems bring their own specific stability issues (Cleland, 1998) in addition to making analysis of the stability of the active ingredient more challenging (Yang et al., 1999).

As discussed above, input from various departments, such as discovery research, pharmacokinetics, toxicology, purification, manufacturing, clinical and marketing, must be considered when deciding on the most appropriate dosage form for a given product. In the past, the drug development process has been more or less sequential, going from research to development to clinical, to manufacturing, and then to marketing. A change in strategy is required where there is more interactive, simultaneous communication between the various drug development departments. With the proper information from the other units, one should rapidly be able to decide on dosage form and develop candidate formulations.

Preformulation Studies

The exquisite sensitivity of protein structure, function, and stability to the primary sequence does not readily lend itself to a generic approach for protein formulation. Therefore, determination of the relative importance of various degradation pathways and elucidation of instability mechanisms for a given protein are essential. Even for closely related proteins, the relative stability and major

Samsung Bioepis Exhibit No. 1002
Page 8039

pathways for degradation might be quite different. Thus, the preformulation studies of new protein entities become very important and crucial tasks in the overall HTF strategy.

Preformulation refers to experimental studies designed at understanding the chemical and physical behavior of the protein or drug candidate. The major focus is on the solubility and stability as a function of a number of extrinsic factors, such as pH, protein concentration, ionic strength, buffer composition and temperature. This information will guide the appropriate choice of excipients, buffers and final protein concentration. Although a number of studies could be envisioned, HTF requires that these studies be very specific and focused. Any information that may narrow the choices would be essential for speedy formulation development. Relevant data can be gathered from the process-engineering group responsible for purification or literature studies on similar proteins, even if it is anecdotal. The final dosage form must also include constraints from marketing, clinical and regulatory groups addressing issues related to eventual approval of a pharmaceutically acceptable formulation for market. Ultimately, all of these data may be required to ascertain the most beneficial composition of excipients and the most appropriate dosage form for the protein product in hand.

Typical time scales for preformulation can range from one to three months depending on what assay systems have been established by the research groups and whether they can be utilized as stability-indicating. Therefore, early involvement of all of the formulation experts is critical for rapid development of lead formulations during the developmental phase. An additional driving force is also the fixed time required to generate the stability data to validate the formulation choice.

Proper Choice of Excipients

Liquid Formulations. If a solution dosage form is indicated, then there will be a finite set of possible excipients, restricting choices to those that are found in approved products and have been shown to be effective in protein formulations. For solution formulations, a list of possible excipients is given in Table 2.

Given that most protein formulations will exist at pH values between 4 and 9, there is a limited set of buffers that will exhibit sufficient buffering capacity. A number have been used in protein formulations and they are listed in Table 2. The main criteria for selection are good buffering capacity at the desired pH and lack of ability to accelerate specific chemical and physical reactions. Some proteins can preferentially bind certain buffer species, altering the conformational stability of the protein. In addition, some buffers (e.g., phosphate, Tris) have been

Samsung Bioepis Exhibit No. 1002
Page 8040

Table 2.
Possible Excipients for Use in Liquid Formulations

Excipient Class	Choices
Buffers	Histidine, Succinate, Acetate, Citrate Phosphate, Tris, Carbonate
Salts	Sodium Chloride, Calcium Chloride Magnesium Chloride
Non-Specific Stabilizers	Sucrose, Trehalose, other sugars, Amino acids (e.g., lysine, glycine)
Specific Stabilizers	depends upon the protein
Surfactants	Tween 20, Tween 80, Pluronic F-68 Sodium Dodecyl Sulfate
Chelators	EDTA

reported to catalyze certain hydrolytic reactions, such as deamidation (Patel et al., 1990).

Because proteins are polyelectrolytes, the ionic strength of the medium can often have an effect on structural stability. The most common salt used in protein formulations is sodium chloride. For an isotonic product, often it is advantageous to keep the concentration of salt as low as possible. Otherwise, the ability to employ stabilizing excipients will be limited.

Structural stabilization of proteins by specific ligands or cofactors in solution formulations can be important for formulation optimization. The mechanism by which these compounds increase thermodynamic stability is described by Timasheff (1992, 1995, 1998) and in other Chapters in this book. Several pharmaceutically important proteins have been stabilized by addition of specific ligands (including metals ions such as calcium), including fibroblast growth factor (Volkin et al., 1993) and DNAse (Chen et al., 1999).

To stabilize proteins (both in aqueous solution and during freezing) with non-specific compounds (e.g., sugars), relatively high concentrations (ca. > 0.2 M) of ligand (solute) are needed to affect protein stability. The mechanism of such stabilization is described in more detail in Chapters 2 and 3. The main point for the current discussion is that in the presence of nonspecific stabilizing excipients fewer protein molecules are unfolded at any point in time, either partially or completely, which reduces the amount of aggregation-competent species and the rate of aggregation (Kendrick et al., 1997, 1998). Addition of small amounts of a specific ligand should theoretically accomplish the same outcome. The most effective non-specific stabilizers tend to be disaccharides, such as sucrose and trehalose. However, certain salts, amino acids, and polymers are preferentially excluded as well. Still, unless there is evidence for advantage in use of a

Samsung Bioepis Exhibit No. 1002
Page 8041

particular compound from this group, sucrose and trehalose should remain the first-line choices.

Finally, one must decide on whether to include a surfactant in the formulation. Surfactants have been shown to inhibit protein aggregation during agitation (e.g., Bam et al., 1995; Krielgaard et al., 1998). The mechanisms by which surfactants stabilize proteins are discussed in a separate Chapter. The surfactant often will be most effective at or above the critical micelle concentration. In Table 2, only the surfactants that have been approved for use in parenteral products in the U.S. have been listed. Note that one potential drawback of including surfactants in the formulation are contaminants (e.g., peroxides) that could cause chemical degradation of proteins (e.g., oxidation).

Lyophilized Formulations. It can be assumed that most proteins will not exhibit sufficient stability in aqueous solution to allow a liquid formulation to be developed. Our understanding of the basic requirements for obtaining a stable lyophilized protein formulation is relatively well developed. The question then is what combination of excipients will allow such a formulation to be prepared. The minimal composition includes a buffer species, an additive capable of forming an amorphous glassy state and inhibiting lyophilization-induced unfolding in which the protein remains entrapped, a bulking agent to provide cake stability, and possibly a surfactant to retard surface-induced damage and/or promote refolding. The rationales for the minimal criteria for a stable lyophilized formation and the choice of appropriate excipients are described in detail in another Chapter.

While a comprehensive formulation development algorithm is yet to be published, following the HTF process outlined above, one can imagine obtaining a rational approach towards selecting final candidate formulation that can be used routinely, even with new classes of therapeutic proteins. Clearly, the details of each case will be guided by the outcomes of the preformulation studies. However, even without a complete data set from the preformulation experiments, implementation of a few standard formulations should be possible. Ultimately, we believe there will be a relatively small number (six of less) of generic, standard lyophilized formulations that will work for nearly all proteins. Each of these will have a corresponding lyophilization cycle that can be used to achieve optimal results. When the HTF process has evolved to this optimal state, the personnel demands be greatly reduced (in terms of full-time equivalents, or FTEs) and the time lines should shrink significantly from the current average of nearly one year.

Estimates of Resources Needed for Formulation Development

The current estimates of the resources required and the possible timelines for formulation development are given in Table 3. Specialized formulations

Samsung Bioepis Exhibit No. 1002
Page 8042

Table 3.
Estimated Resources Needed for Development of Final Stable Formulations of Protein Pharmaceuticals

Resource	Current Status	Initial HTF Targets	Optimal HTF Targets
• Personnel			
Standard Formulations	2 FTE	2 FTE	2 FTE
Complex Formulations	3–5 FTE 3–4 FTE	3 FTE	
• Time Scale			
Standard Formulations	12 months	6–9 months	3–6 months
Complex Formulations	12–24 months	12 months	6–9 months
• Minimal Amount of Material			
Standard Formulations	200–500 mg	100–200 mg	50–100 mg
Complex Formulations	400–2000 mg	200–500 mg	100–200 mg

(suspensions and controlled release dosage forms) will require more resources and less aggressive time lines (see Table 3). However, even this situation should improve as more of these types of products enter the marketplace. We anticipate that a significant number of these types of products will be approved over the next ten years. By then, most large pharmaceutical companies will have acquired the necessary expertise to develop them at rates comparable to the standard formulations of today. Once the HTF scheme has evolved to this stage, a company should be able to develop final formulations quickly and easily, even if personnel levels are relatively constant.

Interestingly, while a streamlined approach towards final formulation selection is the heart of the HTF strategy, it is the one aspect that can be introduced into any current product development scheme almost immediately, as it can be accomplished solely based on the current state of understanding of protein stabilization. Other aspects of the HTF package will demand more time to implement, as they will require significant advances to be made, particularly in the areas of analytical methodology and information technology. These two features of the HTF scheme are described below.

USE OF SOFTWARE AND DATABASES TO ASSIST IN THE HTF PROCESS

Unless formulation scientists have access to all pertinent information, critical choices for formulation components could be delayed or, even worse, incorrect. Therefore, we propose that a key aspect of the HTF process is to develop an integrated information system that can assist the scientist in making critical

Samsung Bioepis Exhibit No. 1002
Page 8043

decisions. Each and every step in the formulation development process is predicated upon having the most recent and comprehensive information on the physicochemical properties of the protein under development, as well as on the effects of additives on protein structure and stability. Clearly, the latter part of this goal is ever evolving as our knowledge of protein chemistry increases. Therefore, the final HTF scheme will involve a central database of essential information. While a large amount of this type of information is currently available, it appears in a multitude of sources, generated by researchers in disparate disciplines, ranging from materials science to biochemistry to food science to pharmaceutics. This makes access to the data difficult. Unless efforts are made to assemble this data in a cohesive package soon, the challenge will become even greater. Once the task is accomplished, sufficient information should be available to predict the performance of certain types of formulations.

In the absence of such resources, one can still make use of existing software and databases. These are summarized below. Briefly, they cover three areas: predictive protein algorithms based upon primary sequence, sequence homology analysis software, and programs aimed at predicting relative stability of proteins. Summaries of the locations and availability of these programs and databases has recently been published (Cook, 1999; Apweiler, 1999).

Predictive algorithms allow one to analyze a primary sequence and predict a variety of chemical and physical properties. For example, there has been enormous effort to develop schemes whereby one can predict the three-dimensional folding of a protein based solely on its sequence. Although there have been some successes, it has been demonstrated in double-blind studies that analysis of new sequences, when they have no clearly related partner in the existing database, fail to provide an accurate prediction of the folded conformation (Orengo et al., 1999; Vencloyas et al., 1999). When the analysis can be guided by sequence homology, the accuracy rises dramatically. Likewise, one can imagine developing structure-stability relationships, based solely upon primary sequence. Some studies of this nature have been reported. For example, Pandit and co-workers relate the frequency of certain dipeptide sequences to instability in proteins (Reddy, 1996; Guruprasad et al., 1990). The result is an algorithm that predicts the relative stability of a protein. Although the degradation criteria are based upon intracellular stability, this approach does provide researchers with an expectation of the overall fragility of the protein in question.

In another example of this type of sequence analysis, Roger et al. (1986) identified regions in proteins that were high in proline, acidic side chains (Asp and Glu), serine, and threonine. The presence of these regions was associated with more rapid degradation *in vivo*. Similar correlations should exist for *in vitro* stability. Even programs to predict likely deamidation sites (where Asn is converted into Asp-like residues) would be helpful, especially as our ability to remove those sites by recombinant DNA technology improves. There is now a

Samsung Bioepis Exhibit No. 1002
Page 8044

solid basis on which to predict both primary and secondary structure effects on deamidation rates (Wright, 1991; Xie and Schowen, 1999), which is considered in more detail in Chapter 4.

Given that numerous protein sequences, whether determined directly or extrapolated from nucleotide data, are available in databases that can be accessed via the world wide web, focused studies generating data on specific instabilities (e.g., deamidation, oxidation, aggregation) could lead to similar structure-stability relationships. The number of available sequences has been doubling every 18–24 months over the last ten years (Cook, 1999). Similarly, there has been an explosion in algorithms and software to analyze these data. Many focus on sequence homology and identification of functional and genetic relationships. Given these advances, it is not unreasonable to expect that stability-indicating profiles could be developed as well.

In addition to stability predictions, many programs are available to calculate important chemical and physical properties such as the isoelectric point, the hydrophobicity profile, the positioning of secondary structure elements, and the conformational flexibility. Knowledge of these properties would help the formulation scientist anticipate the behavior of a new molecule provided for development into a potential drug product. Furthermore, algorithms to predict correlations of these physical properties with physical and chemical stabilities of therapeutic proteins should be straightforward to design and implement with current programs.

ESSENTIAL ANALYTICAL METHODS

One must have access to certain equipment in order to characterize properly protein degradation pathways and effects of formulation variables on protein stability. In addition, we will discuss some areas where technological advancements must be made in order to allow formulation scientists to work with smaller amounts of protein. While a number of reliable and accurate stability-indicating assays exist, there needs to be efforts to miniaturize some of them. This will allow development of proteins where the amount of material is limited, e.g., less than 100 milligrams total.

The first analytical methods that are needed are those that can quantify the protein concentration. As determination of accurate extinction coefficients is straightforward, ultraviolet absorption spectroscopy is inexpensive and preferred over chemical methods, such as the Lowry method. One also needs to be able to monitor the global structure of the protein. Circular dichroism (CD) spectroscopy allows one to follow changes in both secondary and tertiary structure of proteins in solution (Manning, 1994). Infrared spectroscopy can also be used to detect

Samsung Bioepis Exhibit No. 1002
Page 8045

secondary structural changes (see below), and fluorescence and derivative U spectroscopies can be used to study tertiary structure.

The second category of required analytical methods are those that monitc retention of native primary structure, namely, chromatographic methods, suc as reversed phase HPLC and ion exchange HPLC, or alternative methods lik capillary electrophoresis. Among the most useful applications of these HPL methods is the generation of a "tryptic map", which is accomplished by digest ing the protein with a sequence-specific enzyme (e.g., trypsin) and separating th resulting peptide fragments by reversed phase HPLC. The pattern then become a sensitive fingerprint of the protein. Any chemical modification of the protei will cause one or more of the peaks to shift, indicating damage to the protein Mass spectrometry can be used to determine the specific chemical modificatio leading to the peak shift.

Among the most important degradation pathways for proteins is aggrega tion. A number of methods are available to characterize and quantify aggregatio levels in proteins, including polyacrylamide gel electrophoresis (PAGE), siz exclusion chromatography (SEC), light scattering techniques and analytical ultra centrifugation. Although each of these methods has its advantages and disad vantages, all four should be considered essential, as they can be complementary They allow both qualitative and quantitative characterization of the soluble an insoluble aggregates in a bulk substance or final formulation.

For lyophilized formulations, a certain set of equipment is considered to b the minimum required for conducting formulation development properly. Thes include an infrared (IR) spectrometer, a Karl Fischer titrator, a differential scan ning calorimeter (DSC), and x-ray diffractometer. The IR instrument will allow one to determine the extent of structural damage (at a secondary structure level in the solid state. In addition, it can be used to monitor the secondary structur composition in the original solution (prior to lyophilization), in the frozen soli and in the reconstituted product (Carpenter et al., 1998). X-ray diffraction c solids allows one to determine the crystallinity of a solid sample. In formulation where the bulking agent is crystallizing during the lyophilization process, on needs to know whether the crystallization is complete. Incomplete crystallizatio can lead to degradation during storage (cf. Carpenter et al., 1997). The Ka Fischer apparatus allows one to measure the amount of water in the final cak Typically, moisture contents need to be at 1–2 % in order for the product to hav the optimal glass transition temperature (T_g) and maximal stability. Finally, DS is required to measure the T_g of the final formulation. The storage temperatur must below the T_g. As the minimal acceptable storage temperature for lyophilize formulations is room temperature, a minimum T_g would be about 40° C. In add tion to these instruments, access to a microscope equipped with a lyophilizatio stage is valuable.

The issue of how to develop formulations with limited amounts of materi is a critical concern. One can imagine adapting a number of existin

Samsung Bioepis Exhibit No. 1002
Page 8046

technologies to assist in assessing the viability of a given formulation while using very small amounts of material. These would include:

1. microtiter plate assays,
2. robotics to assist in sample preparation and analysis,
3. ELISA or other antibody-based recognition assays for intact drug,
4. multi-sample adaptations of existing analytical techniques (e.g., DSC or analytical ultracentrifuge)
5. nanoseparation methodology (the so-called "lab-on-a-chip" approach),
6. surface-immobilized analytical methods, such as the BIAcore.

Each of these methods could provide significant increases in the capability to monitor the stability of a protein in a microformulation (less than 10 μg of active drug). For example, new chip technologies (either DNA- or protein-based) are becoming powerful tools that are increasingly used in all areas of drug discovery. They offer significant advantage over older technologies in terms of speed, sensitivity and cost. It is important to consider the use or implementation of these tools in formulation studies. One can envision protein chips doing for the study of proteins what DNA chips have done to the study of genes. That is, they can be used to identify critical parameters required for rapidly developing a stable formulation. Similarly, any of the other methods could be modified to assist the formulation scientist.

Finally, there has been a steady improvement in protein analysis tools such as mass spectrometry and more powerful and versatile biochemical techniques, which are not only more sensitive but offer the advantages of speed and low cost. So with the emerging technological advances in analytics and implementation of these enabling technologies in preformulation and formulation studies, one can envision a more time and resource efficient process for developing stable protein pharmaceuticals. Most of these technologies are being developed to meet the needs of efficient drug discovery research. The challenge here will be the speed at which the formulation scientists adapt these technologies into their programs. Unfortunately, these tools have not yet reached their maturity, so the implementation of micro-analytical methods for preformulation and formulation is crucial for developing HTF strategies for protein pharmaceuticals.

STABILITY PROTOCOLS

In addition to formal storage stability protocols that would be acceptable to regulatory agencies for real-time data, one must have standard approaches for assessing the possible long-term fate of the candidate formulations. Given the aggressive time line of the HTF strategy, one must rely, to some degree, upon accelerated storage studies to guide decision about suitable excipients. These

Samsung Bioepis Exhibit No. 1002
Page 8047

could be done as isothermal studies, storing the protein at room temperatu and 40° C, for example. In certain cases, exposure to higher temperature such as 50° C or even 60° C, may be warranted. However, it is important to no that at higher temperatures, there is the distinct possibility that the rat determining pathway may change compared to that at the projected stora conditions.

An alternative to isothermal testing that needs to be considered is the use non-iosothermal stress testing. In this procedure, the sample is exposed to a line increase in temperature over time (or approximately linear with respect to time Samples are taken at set intervals and assayed for protein damage. Using an alg rithm developed by Lee and Stavchansky (1998), it is possible to determine t Arrhenius parameters in a single experiment, which would otherwise require least three separate isothermal studies. This approach has been demonstrated f a pentapeptide (Lee and Stavchansky, 1998), and should be extended to larg proteins in the future.

UNIFIED STRATEGY FOR HTF

The requirements for HTF can be summarized in four points. First, integ tion of speed, sensitivity and cost can be achieved by having in place go analytical methodologies for monitoring both chemical and physical instabil mechanisms. These methods can serve as tools for elucidating the mechanism instability of protein drugs and also as stability indicating assays during analy of the final formulation. Second, identification of the instability mechanism proteins can lend itself to developing a fast-track stable liquid protein formu tion, although the disadvantages of addressing biocompatibility and transpor tion issues have to be addressed with such dosage forms. A third requirement HTF is to have obviously a safe and a convenient dosage form for administ tion. Coupled with this criterion should be the requirement for manufacturi the formulation using standard processes, so that if needed the production can out-sourced to a contract facility.

Finally, technological advances will be a major driver for HTF and in gene biotechnology development. The ability to work at low microgram to milligr scale of protein is essential for the ability to start the process before the d candidate achieves development status. In many cases, companies may initia neglect formulation and stability issues that will haunt them in later stages development. The development of successful formulations is dependent u the ability to study both the *in vitro* and *in vivo* characteristics of the prot drug, as well as its intended application in the clinic. Thus, it is essential the formulation group to work closely with the discovery research,

Samsung Bioepis Exhibit No. 1002
Page 8048

pharmacokinetics and the toxicology departments prior to the decision to move the drug into full-scale development. Resolving the difficulties in the early research and development stages will not only help in targeting the appropriate drug candidate for development, but also speed up the selection of a pharmaceutically acceptable formulation for clinical trials. What we propose is the involvement of the formulation group in a multi-disciplinary environment where interactions start early in the research environment. The appropriate lead candidates would be selected not only based on efficacy studies, but also on pre-formulation studies. We envision these studies to take one to three months to establish appropriate profiles for formulation development. As a result, the lead formulation can have the advantages of demonstrating efficacy and potential stability of the protein drug.

During the development process of a biotechnology drug we envision formulation to take typically three to six months. This period would encompass preformulation studies and evaluation of the formulation options determined by the planned clinical studies. Hence, protein formulation and purification can be developed concurrently using limited quantities of the product, and identification of the lead formulation could occur at the time of establishment of the purification process. What we propose is not a sequential development pathway for protein drugs, but a concurrent scenario where formulation design is initiated very early in the drug development pipeline. We are quite confident that the HTF can be coupled successfully with high throughput screening strategies of protein drugs and facilitate drug development within the inherent time and resource constraints of the pharmaceutical industry.

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Page 8049

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Samsung Bioepis Exhibit No. 1002
Page 8052

Index

Acid-soluble spore proteins (SASP), 97–98
Acidic fibroblast growth factor (aFGF), 69
Administration, route of, 182
Adsorption, 13, 152, 162–164
Aggregation
  covalent and non-covalent, 13
  nonionic surfactants to inhibit, 127
Agitation, and stability, 71–72
Analytical instruments, 3
Analytical methods, essential, 180, 191–193
Antibodies: *see* Recombinant humanized anti-IgE monoclonal antibody
ArgHCl, 45–46
Arrhenius equation, 8
Asparagine (Asn) residues, deamidation of in peptide and proteins, 86–92

Bax, 169
Bioactivity, 49
Bovine pancreatic trypsin inhibitor (BTPI), 40
Bovine serum albumin (BSA), 154–155
Bovine somatotropin (BSTI), 91
Büchi laboratory spray dryer, 139–141, 146, 147
Buffers/salts, 14, 67
  choice of, 118–119
  debye screening, 67–68
  ionization enthalpy, 68
  lysis, 33
  preferential exclusion of salts, 67
Bulking agent, 14, 126
Carbohydrates, to stabilize spray-dried proteins, 149
Chemical modifications, 17
Chloramine T, 98
Chromatography, 192
  column, 7
Cleavages, 13
Commercial formulation, 19
  for early preclinical and clinical studies, 19
Commercial formulation development (process), 18
  decisions during, 18–19
  regulatory issues in, 20–22
  timeline, 18
Conformational stability, 51
Cosolvents, preferentially excluded, 66–67
Critical micelle concentration (CMC), 164, 165, 171
Cyclic imide, 13
Cytokine receptor homology (CRH), 30
Deamidation, 13; *see also under* Polypeptides, structure of
Decision tree, role of established for formulation design, 181–182
Degradation
  chemical and physical mechanisms, 160
  conditions used to accelerate, 6
  enzymatic, of non-ionic surfactants, 170
  minimization of routes of chemical, 116–117
Degradation products, qualification of, 17
Dehydration-induced unfolding, mechanism for inhibition of, 123–124
Delivery of products, 16
Denaturants, removal of, 41–43
  solid phase, 43
Denaturation, 13, 70–72
Deuterium, 96–97
Dialysis, 41–42
Dilution, 42–43
Disaccharides, 149
Disaggregation, high pressure, 48
Disulfide bond analysis, 50
Disulfide bond formation, 41

Samsung Bioepis Exhibit No. 1002
Page 8053

Dosage forms
  design of successful, vii–viii
  proper choice of, 183–185
  unconventional, 17
Drug delivery systems, specialized, 185
Drug design, rational, vii
Drying-air volumetric flow rate (v_{da}), 147
Electrophoresis, 7
Equilibrium unfolding, 62
Erythropoietin, recombinant human, 137
Escherichia coli (*E. coli*)
  deamidation of histidine-containing protein in, 90
  recombinant production of native proteins from, 27–28
    cell washing and lysis, 32–34
    distribution of expressed proteins, 28–31
    methods to analyze folded structures, 48–51
    purification and refolding of proteins from inclusion bodies, 36–37
    purification and refolding of soluble, misfolded proteins, 35
    purification of expressed proteins from inclusion bodies, 36–37
    purification of soluble, folded proteins, 34–35
    refolding mechanism, 38–40
      disulfide bond formation, 41
      effects of excipients, 44–47
      effects of tag sequences, 44
      high pressure disaggregation and refolding, 48
      removal of denaturant, 41–43
      response surface methodology, 47–48
Excipients, 21, 170
  animal-derived, 15
  choice of, 117, 120–121, 186–188
  effects on refolding mechanism, 44–47
  interactions with proteins, 65, 66–70
    preferentially excluded cosolvents, 66–67
  limiting solvent accessibility, 96

Fibroblast growth factor (FGF), 31, 32, 69
Fibronectin, 98
Formulation change/amendments, results required to file, 20–22
Formulation development, 1–2, 10; *see also specific topics*
  estimates of resources needed for, 188–189
Formulation development (*cont.*)
  financial ramifications of rapid *vs.* delayed, 110–111
  necessary studies for, 15–16
  preformulation development, 4–5
  preparation for
    resource requirements, 3–4
    useful information for designing formulations, 4
Formulation optimization studies, design of, 15
Formulation options for protein pharmaceuticals, 10
  liquid formulations, 10–11
  single- and multidose forms, 12
  solid dosage forms, 11–12
Formulation problems, strategies to overcome, 17
Formulation studies, information useful for, 4
Formulation variables, optimization of, 13–15
Formulations
  constraints on pharmaceutically acceptable, 182–183
  important components, 13–15
Free energy *vs.* extent of reaction, 73
Freeze-drying microscopy, 115
Freeze-thawing, and stability, 71, 125
Freezing protection, mechanism for, 121–123
Frozen formulations, 183–184
GdnHCl, 35, 36, 45
Gel filtration, 43
Gibbs adsorption equation, 162–164
Glass immobilization hypothesis, 149, 151
Glass transition temperature (T_g), 113–116, 120, 125, 126
Glutathione S transferase (GST), 44
Glycine, 116, 126, 150
Granulocyte colony stimulating factor (G-CSF) receptor, 29
Growth factor
  fibroblast, 31, 32, 69
  keratinocyte, 31, 32, 35
Growth hormone, recombinant human, 137, 143, 147–148, 152–154, 167, 170, 171
Growth hormone-releasing factor (GRF), 90
Handling of products, 16
High-pressure liquid chromatography (HPLC), 192
High throughput formulation (HTF), 178–179, 186, 188, 189
  concept, elements, and structure of, 179–181

Samsung Bioepis Exhibit No. 1002
Page 8054

High throughput formulation (HTF) (*cont.*)
requirements for, 194
software and databases to assist, 179, 189–191
unified strategy for, 194–195
Histidine-containing protein (HPr), 90
Hydrogen to deuterium (H-D) exchange rates, 96–97
Hydrolysis of peptides, 85; see also Deamidation
Hydrophobicity and hydrophobic interactions, 62–63, 72, 95, 161, 163, 166
Hydrophobicity reversal, 167–168
Hydroxyethyl starch, 126
Impurities, 14–15
Inclusion bodies (IBs), 29, 36–37
Infrared (IR) spectroscopy, 191–192
Interleukin-1$ (IL-1$), 28–30
Interleukin 6 (IL-6), 40, 50, 51
I(SH) and I(SS), 39, 41
Isothermal and non-isothermal stress testing, 194
Keratinocyte growth factor (KGF), 31, 32, 35
Lactate dehydrogenase (LDH), 138, 144, 145, 154, 155
Lactose, 149, 150
Ligands, 119–120
non-specific, 96
specific binding, 68–69
Light scattering/turbidity, 7
Liquid formulations, 10–11, 184
excipients for use in, 186–188
Lyophilization-induced protein unfolding, inhibition of, 112–113
Lyophilized products/formulations, 11, 109–110, 184, 188
minimal criteria for successful, 111–117
minimization of routes of chemical degradation, 116–117
storage below formulation glass transition temperature, 113–114
strong, elegant cake structure, 114–116
water content relatively low, 114
rational design of stable, 117–118
bulking agent, 126
choice of buffer, 118–119
ligands/pH optimizing thermodynamic stability of protein, 119–120
trehalose/sucrose to inhibit protein unfolding and provide glassy matrix, 120–126
Lyoprotectants, 149
Lysis buffers, 33
Maltose binding protein (MBP), 44
Mannitol, 116, 126, 137–138, 149–151
Manufacturing process, 21
Metal-catalyzed oxidation (MCO), 94–95
Metals, trace, 94
Methionines and methionine residues, 94–100, 116–117
Micelle concentration, critical, 164, 165, 171
Microcharacterization methods, 7
Monoclonal antibodies: *see* Recombinant humanized anti-IgE monoclonal antibody
Murine IFN-((mIFN-(), 37–38
Mutagenesis, site-directed, 17
OmpA, OmpG, and OmpT proteins, 28–29
Optimization of formulation variables, 13–15
Osmotic pressure, 165
Ovalbumin, 40
Oxidation (processes), 13; *see also* Polypeptides, structure of, role in protein oxidation
excluded effects on, 98–99
metal-catalyzed, 94–95
non-site-specific, 94–97
Oxygen, singlet ($^1O_2$), 93
Packaging material, 21
Parathyroid hormone, 95
Peptides: *see* Polypeptides
Periplasmic secretion strategy, 28–29
Peroxides, organic, 93
pH, 14, 186
Polyethylene glycol (PEG), 46
Polypeptides, structure of, 85–86, 101
deamidation rates and, 86–87, 92
primary structure effects, 87–89
secondary structure effects, 89–91
tertiary structure effects, 91–92
role in protein oxidation, 92–93, 100–101
conformational control of oxidation in aqueous solution, 97–99
effects of oxidation of methionines on protein structure, 95–96
limiting solvent accessibility of residues, 96–97
structural control of oxidation in lyophilized products, 99–100
types of oxidation processes, 93–95
Polysorbate concentration, critical, 153, 154

Samsung Bioepis Exhibit No. 1002
Page 8055

Preferential exclusion mechanism, 122–123
Preformulation development, 4–10
  development of analytical methods, 6, 7
  evaluation of the significance of problems, 7–8
    qualitative assessment, 9–10
    quantitative assessment, 8–9
  information obtained from, 5
Preformulation studies, 185–186
Preservatives, 12
Pressure, and stability, 72
Product development timeline, 18
Protein formulation: *see* Formulation
Protein pharmaceuticals, characterization of, 5
Proteins; *see also specific topics*
  maintenance of biophysical and biochemical properties of, 16
  typical methods used to characterize, 6, 7
Proteolysis, 85
  limited, 51
Purified protein, 3
Purity of raw materials, 14–15
Reaction, free energy *vs.* extent of, 73
Receptor binding, 49
Recombinant human erythropoietin (rhEPO), 137
Recombinant human growth hormone (rhGH), 137, 143, 147–148, 152–154, 167, 170, 171
Recombinant humanized anti-IgE monoclonal antibody (rhuMAbE25), 137–138, 148, 150–151
Regulatory applications, information included in, 20, 21
Regulatory documents, 22–23
Regulatory guidelines, 2
Regulatory license for drug product, results required to apply for, 20
Residues; *see also* Methionines and methionine residues
  asparagine (Asn), 86–92
  solvent accessibility, 96–97
Response surface methodology (RSM), 47–48
Salts: *see* Buffers/salts
SASP (acid-soluble spore proteins), 97–98
Sequence analysis, 190
Serine hydroxymethyltransferase (SHMT), 91
Sodium chloride (NaCl), 118
Sodium dodecyl sulfate (SDS), 160
Solid dosage forms, 11–12
Solubilizer, 14
Solutes, excluded, 99
Solvent accessibility of residues, 96–97
Spectroscopy, 7, 50–51, 191–192
Spray-drying of proteins, 156
  conditions, 140
    drying air volumetric flow rate, 147
    liquid feed rate and atomising air volumetric flow rate, 146–147
    temperature (T_{inlet}, T_{outlet}), 140–146
  developments in last 10 years, 136–138
  equipment, 139–140
  formulated systems, 149–151
  influence of formulation, 147
  pure proteins, 147–149
  reasons for, 135–136
  use of added surface active substances, 151–156
Stability (physical), protein, 61–62, 73, 181
  kinetic control of, 63–65
  physical factors affecting, 70–72
  self-stabilization, 69–70
  thermodynamic control of, 62–63
Stability problems, typical
  causes and solutions, 13
Stability protocols, 193–194
Stability studies, 3
  accelerated, 5–6
  guidelines for, 20
  storage, 15–16
Stabilization, structural, 187
Stabilizers, 14
  non-specific, 96, 187
Storage stability studies, 15–16
Stress testing, non-isothermal, 194
Sucrose, 66, 113, 114, 120–121, 138, 149, 150; *see also under* Unfolding
Sugar; *see also* Sucrose
  rationale for choice of stabilizing, 120–121
Surface denaturation, 13
Surface tension, 165
Surfaces, proteins and surfactants at, 161–166
Surfactant-protein interactions, 159–160
  in solution, 166–167
Surfactants, 188
  effects on protein assembly state, 167–169
  effects on proteins during freezing, freeze-drying, and reconstitution, 169–170
  non-ionic, 160, 161
    enzymatic degradation of, 170

Samsung Bioepis Exhibit No. 1002
Page 8056

Surfactants (*cont.*)
nonionic, to inhibit aggregation, 127
recommendations for protein formulation, 170–171
at surfaces, 161–166

Tag sequences, 44
Temperature, and stability, 70–71
Tetrapeptides, 90
Thermal analysis, 7
Thermodynamic box, classical, 77–78
Timasheff preferential exclusion mechanism, 73–78
Tonicity, 182
Tonicity modifier, 14
Transportation of products, 16
Trehalose, 113, 120–121, 149, 150, 154, 155; *see also under* Unfolding
Trypsinogen, 138, 148
Turbidity, 7
Tween 20, 164–170
a/b-type acid-soluble spore proteins (a/b-SASP), 97–98

Unfolding
dehydration-induced, 123–124
equilibrium, 62
lyophilization-induced, 112–113
trehalose/sucrose to inhibit, 120–126

Water replacement hypothesis, 149, 151
Water replacement mechanism(s), 123–124
Wide-angle X-ray diffraction (WAXS), 150
Wyman linkage function, 73–78

Samsung Bioepis Exhibit No. 1002
Page 8057

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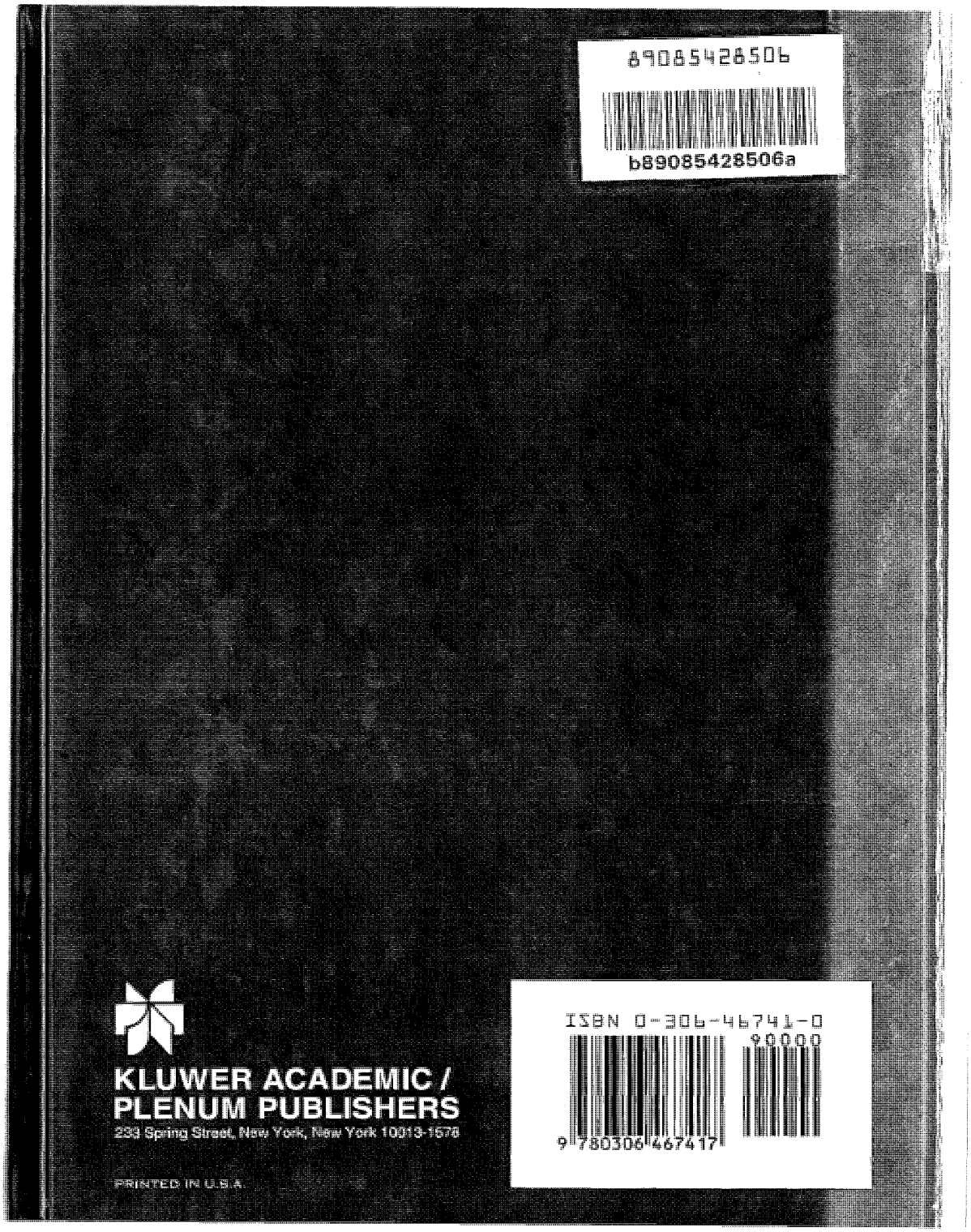
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(Application No. 11001632.6)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C411599EP PXT/ALS/hdb

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Proprietor/representative's reference	C411599EP

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Application No.	EP11001632.6
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor of the patent	ALEXION PHARMACEUTICALS, INC.

Documents attached:

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1	Any annexes (other than citation) to an opposition letter - Letter enclosing an updated Consolidated List	Letter Enclosing Updated Consolidated List C411599EP.PDF	OTHER-1.PDF
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Date:	**14 December 2018**
Signed by:	**Amanda Simons 23469**
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Document Name	Consolidated Number	Patentee	Opponent 1 (Amgen)	Opponent 2 (Hoffman Eitle)
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Samsung Bioepis Exhibit No. 1002
Page 8064

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Samsung Bioepis Exhibit No. 1002
Page 8065

Rituximab 1997 Product Data Sheet	D47		D44	
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Proprietor	ALEXION PHARMACEUTICALS, INC.
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Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

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WO 2005/092073 A2	D52			D42
WO 2006/003179 A2	D53			D43

Samsung Bioepis Exhibit No. 1002
Page 8073

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Maier, Dagmar

Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastrasse 30
81925 München
ALLEMAGNE

Date
21.12.2018

Reference	Application No./Patent No.
198 441 u6/ne OPPO 02	11001632.6 - 1112 / 2359834
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

BRIEF COMMUNICATION

Subject:
- ☐ Your letter of
- ☐ Our telephone conversation of
- ☐ Communication of
- ☐

Enclosure(s):
- ☒ Letter from the proprietor of the patent of 14.12.2018
- ☐ Letter from the opponent of
- ☐ Copy (copies)

Communication:

☐ A letter from the opponent /proprietor was received on ..... . The documents specified as patent documents in this letter are available via the European Patent Register at **www.epo.org/register.** Should you wish to receive these documents in paper format, they will be supplied to you free of charge if specifically requested on receipt of this communication (OJ EPO 2009, 434).

☐

For the Opposition Division

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☒ **Registered letter**

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Page 8074

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The European Patent Office
Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

14 December 2018

Dear Sirs

Opposition to European Patent No. 2359834
(Application No. 11001632.6)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C411599EP PXT/ALS/hdb

Following the submissions made by the various parties in November, a number of new documents have been filed. In order to assist the Opposition Division, we enclose herewith a revised consolidated documents list which renumbers all of the new documents as D39 to D53. Please note that the document cited by Opponent 2 as D40 (Mueller et al, 1997, Mol Immunol 34(6) 441-452) is already in proceedings as document D14 so has not been given a new reference number.

I understand from a discussion with the formalities officer that this list will be used by the Opposition Division during oral proceedings. Please let me know, however, if an alternative document numbering system is to be used.

Yours faithfully

Electronically Signed
AMANDA L SIMONS
ASSOCIATION NO. 811

PATENT ATTORNEYS • TRADE MARK ATTORNEYS
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Consolidated List of Documents cited in opposition to EP2359834 B1				
Document Name	Consolidated Number	Patentee	Opponent 1 (Amgen)	Opponent 2 (Hoffman Eitle)
US 2005/191298 A1	D1		D1	D1
Hillmen *et al.*, (2003) *J. Hematol.* 121 (Suppl. 1): 87-95	D2		D2	D2
Hillmen *et al.*, (2004) *N. Engl. J. Med.* 350(6): 552-559	D3		D3	D3
Harding. (2004) *Drugs Future*. 29(7): 673-676	D4		D4	D4
US 2005/221382 A1	D5		D5	D5
Hill. (2005) *Clin. Adv. Hematol. Oncol.* 3(11): 849-50	D6		D6	D6
Morris *et al.*, (1992) *South. Med. J.* 85(19): 1030-1031	D7		D7	
Corvini *et al.*, (2004) *Ann. Allerg. Asth. Immunol.* 93(2): 200-205	D8		D8	
Sierra *et al.*, (1991) *NIPH Annals.* 14(2): 195-210	D9		D9	
US Pharmacopeia (USP29-NF24), page 2989	D10		D10	
Adis Data Information. (2007) *Drugs R. D.* 8(1): 61-68	D11		D11	
Hill *et al.*, (2005) *Blood.* 106(7): 2559-2565	D12		D12	
Thomas *et al.*, (1996) *Mol. Immunol.* 33(17/18): 1389-1401	D13		D13	D8
Mueller *et al.*, (1997) *Mol. Immunol.* 34(6): 441-452	D14		D14	D40
WO 97/11971 A1	D15		D15	
WO 2005/007809 A2	D16		D16	
US 6,355,245 B1	D17		D17	D7
Hillmen *et al.*, (2006) *N. Engl. J. Med.* 355(12): 1233-1243	D18		D18	
EMEA, CPMP, 20 February 2003, *Note for guidance on excipients, antioxidants and antimicrobial preservatives in the dossier for application of marketing authorization of a medicinal product.*	D19		D19	
Wang *et al.*, (2007) *J. Pharm. Sci.* 96(1): 1-26 (January 2007)	D20		D20	D10
Daugherty *et al.*, (2006) *Adv. Drug Del. Rev.* 58: 686-706	D21		D21	
US 2005/0271660 A1	D22		D22	
Expert Declaration by Dr Michael Treuheit	D23		D23	

Samsung Bioepis Exhibit No. 1002
Page 8076

Soliris®(eculizumab) *Highlights of prescribing information.*	D24		D24	
US 2012/0230982 A1	D25		D25	
WO 2007/002571 A2	D26		D26	
Hill *et al.*, (2004) *Blood.* 104: 2823-2824	D27		D27	
US 2003/0202972 A1	D28			D9
US 6,171,586 B1	D29			D11
Maloney *et al.*, (1997) *Blood.* 90(6): 2188-2195	D30			D12
Commercial monoclonal antibodies 2006 (all modes of administration)	D31			Annex I
Commercial monoclonal antibodies 2006 (I.V. administration)	D32			Annex II
Declaration of Dr Miano	D33	D33		
Rouet *et al.*, (2014) *FEBS Letters.* 588: 269-277	D34	D34		
Summary of antibodies listed in D20/D21 Tables	D35	D35		
Graphical representation of D20 Table 2	D36	D36		
Summary of antibody products approved by EMA since filing date	D37	D37		
Extracts from Kabat *et al.*, Sequences of Proteins of Immunological Interest, US Dept, Health and Human Services (1991)	D38	D38		
Declaration of Dr Alexander Klibanov	D39	D39		
Rational Design of Stable Protein Formulations, Pharmaceutical Biotechnology Volume 13, 2002 Kluwer Academic/Plenium Publishers; Chapters 1, 5 and 8.	D40	D40		
Alberts et al, Molecular Biology of the Cell, Third Edition, 1994, page 585.	D41	D41		
Tacken, P.J. et al. Blood 2005, 106(4), 1278-1285;	D42		D39	
WO 2004/050017 A2 (Bowdish, K. S. et al., June 17, 2004)	D43		D40	
WO 2004/108158 Al (Rother, R. P. et al., December 16, 2004)	D44		D41	
US 6,407,213	D45		D42	
Emery et al., Arthritis & Rheumatism 2006, 54(5), 1390-1400	D46		D43	

Samsung Bioepis Exhibit No. 1002
Page 8077

Rituximab 1997 Product Data Sheet	D47		D44	
Declaration by Prof. Ronald Taylor, including CV (Appendix A)	D48		D45	
Hillmen et al, N. Eng. J. Med. 1995, 333:1253-8.	D49		D46	
ICH Harmonised Tripartite Guideline on Stability Testing of Biological Products	D50			D39
WO 2004/071439 A2	D51			D41
WO 2005/092073 A2	D52			D42
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14 January 2019

URGENT ORAL PROCEEDINGS 21 JANUARY 2019

Dear Sirs

Opposition to European Patent No. 2359834
(Application No. 11001632.6)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C411599EP PXT/ALS/hdb

I write with reference to the oral proceedings scheduled for 21 January 2019 and request admission of a new document (referred to herein as D54) into the proceedings. This letter and the enclosed document are also being sent directly to the two Opponents.

D54 is a second declaration of Dr Miano which provides the results of an assay to determine the potency of aggregated eculizumab. This declaration is filed in direct response to Opponent 1's submission of 21 November 2018 which stated in paragraph 18 that:

> *"there is no evidence of a correlation between aggregation and loss-of-activity for this specific antibody!".*

It is the proprietor's position that the skilled person in the art would generally be aware that aggregation is detrimental to the activity of biologic drugs. For instance, aggregation can affect the availability of binding sites. It is therefore generally accepted in the art that aggregation should be avoided. Eculizumab is no exception to this general principle.

However, in case there is any doubt over this matter, Dr Miano reports in D54 the result of a study into the potency of aggregated eculizumab. This confirms that the aggregated form has 50% of the C5-binding activity of eculizumab itself.

We appreciate that this new document is being filed after expiry of the Rule 116 deadline. However, we request the Opposition Division's discretion in admitting this document into proceedings.

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Page 8079

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Yours faithfully

Electronically Signed
AMANDA L SIMONS
ASSOCIATION NO. 811

Samsung Bioepis Exhibit No. 1002
Page 8080

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Submission in opposition proceedings

Representative:

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- representing the proprietor(s):	ALEXION PHARMACEUTICALS, INC.
Proprietor/representative's reference	C411599EP

The information given below is pertaining to the following patent in opposition proceedings:

Patent No.	EP2359834
Application No.	EP11001632.6
Title of the invention	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement
Proprietor of the patent	ALEXION PHARMACEUTICALS, INC.

Documents attached:

	Description of document	Original file name	Assigned file name
1	Any annexes (other than citation) to an opposition letter - Enclose new document D54	Enclose new document C411599EP PXT ALS.PDF	OTHER-1.PDF

Evidence filed subsequently:

D54	Other evidence	Second Declaration by Dr Miano original file name: D54 Second Declaration Dr Miano 14 Jan 19 C411599EP ALS PXT.PDF attached as: Other-evidence-1.PDF

Signatures

Place:

Date:	**14 January 2019**
Signed by:	**Amanda Simons 23469**
Association:	**J A Kemp**
Representative name:	**/Amanda Simons/**
Capacity:	**(Representative)**

IN THE MATTER OF
European Patent No. EP 2 359 834

SECOND DECLARATION OF DR. DINO MIANO

I, Dr. Dino Miano, hereby declare as follows:

1. I am Dr. Dino Miano of Alexion Pharmaceuticals, Inc., 100 College St, New Haven, Connecticut, USA. I am the same Dr. Dino Miano that submitted a declaration known as D33 in these proceedings. Details of my background and my CV were provided with that earlier declaration.

2. I understand that one of the Opponents in this opposition has indicated that no correlation has been shown between aggregation and loss of activity for eculizumab. I was therefore asked to comment on the activity of the aggregate (dimer) form of eculizumab.

3. In 2005 we carried out a number of analyses on the eculizumab dimer sample isolated from eculizumab drug product lot 5000-001A according to according to DVL-05-100-00, "Isolation of Eculizumab Dimer and Monomer." Dimer isolation was performed by manual collection of peaks eluting from a size exclusion column. Collected peaks were pooled and concentrated with a Centriplus YM-10 centrifugal filter device.

4. These analyses included a measure of the C5 binding assay of the dimer. The C5 binding assay determines potency by measuring the ability of eculizumab samples to bind to C5 proteins. The method used for the C5 binding assay was the BioVeris Detection Analysis of C5 binding to eculizumab (Alexion ref BTM-0018.01). The assay uses an electrochemiluminescent (ECLM) reaction and photon detection technology (measured at λ = 620 nm) to determine potency of eculizumab by testing the ability of an eculizumab sample to bind C5 proteins. A ruthenium ion, necessary for the ECLM reaction, is conjugated to C5, a human complement protein. The C5 protein and eculizumab sample are incubated in a microtiter plate. rProtein-L, a biotinylated protein, is added and incubated with the C5/eculizumab complex. After the incubation, magnetized streptavidin beads are added to the plate. The antibody complex attaches to the beads through a streptavidin-biotin association. After incubation with the beads, the sample plate is measured for ECLM detection with a BioVeris system.

5. The purity of drug product lot 5000-001A, as determined by size exclusion chromatography (SEC), was 98.8% monomer and 1.2% aggregate at release (T=0, 01JUN01). This lot was stored at 2-8°C for 52 months for use in this study. The purity after 52 months was determined to be 97.25% monomer and 2.75% aggregate. The aggregate was isolated as described in paragraph 3 above.

6. The aggregate (dimer) peak isolated from drug product lot 5000-001A contained

approximately 95% dimer and 5% monomer when initially tested by SEC; refer to Figure 1.

7. The C5 binding assay was carried out according to the method described in paragraph 4 above for both eculizumab drug product lot 5000-001A and the isolated dimer sample. The source material, drug product lot 5000-001A, had a C5 binding capacity of 92.6%. The isolated dimer sample had roughly half the potency of lot 5000-001A, with a C5 binding capacity of 45.3%. A placebo (lot 2000-001A) was also tested and was below the limit of quantitation in this assay.

8. These results demonstrate that the isolated dimer has approximately 50% of the C5 binding activity of the drug product, indicating a significant drop in potency on aggregation to the dimer form.

I believe and do hereby declare that the facts stated above are to the best of my knowledge true.

Dr. Dino Miano

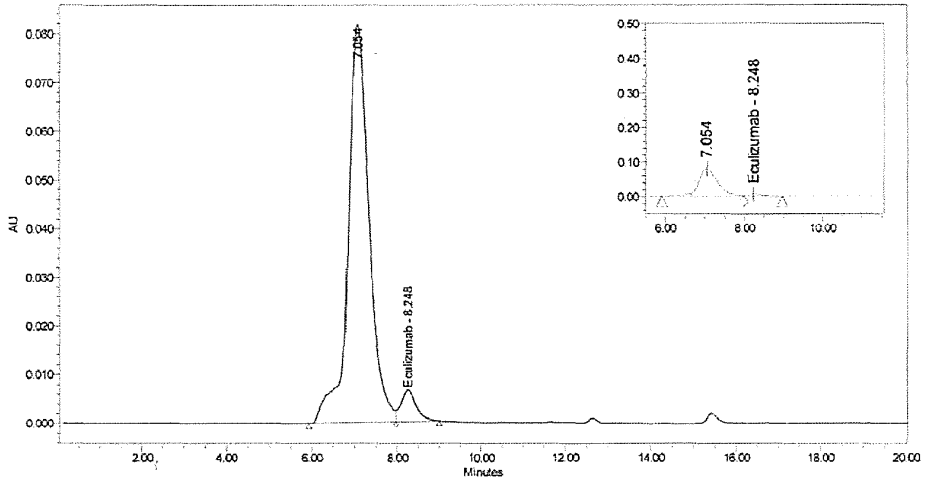
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Samsung Bioepis Exhibit No. 1002
Page 8084

Figure 1: Size Exclusion Chromatography of isolated dimer

SAMPLE INFORMATION

Sample Name:	Concentrated Dimer (26JUL05)	Acquired By:	tineg
Sample Type:	Unknown	Date Acquired:	7/27/2005 11:47:18 AM
Vial:	2	Acq. Method Set:	Eculizumab SEC
Injection #:	1	Date Processed:	7/27/2005 1:53:16 PM
Injection Volume:	43.00 ul	Processing Method	Eculizumab SEC
Run Time:	20.0 Minutes	Channel Name:	Extract 214nm
Sample Set Name:	27JUL05GT01	Label:	



Peak Result Information

	Peak Name	RT(min)	Area (uV*sec)	Height (uV)	Int Type	% Area	Tailing @ 5%	Plate Count	USP Resolution
1		7.054	2795159	61667	BV	94.43	0.882	1179	
2	Eculizumab	8.248	164763	6579	VB	5.57		2358	1.513

Samsung Bioepis Exhibit No. 1002
Page 8085

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Acknowledgement of receipt

We hereby acknowledge receipt of the following submission by the proprietor:

Submission number	7089311
Application number	EP11001632.6
Patent number	EP2359834
Date of receipt	14 January 2019
Your reference	C411599EP
Proprietor	ALEXION PHARMACEUTICALS, INC.
Title	Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement

Documents submitted	package-data.xml ep-oppo.pdf (2 p.) Other-evidence-1.PDF\D54 Second Declaration Dr Miano 14 Jan 19 C411599EP ALS PXT.PDF (3 p.)	ep-opposition-data.xml OTHER-1.PDF\Enclose new document C411599EP PXT ALS.PDF (2 p.)

Submitted by	CN=Amanda Simons 23469
Method of submission	Online
Date and time receipt generated	14 January 2019, 18:27 (CET)
Message Digest	08:8B:89:8A:67:17:76:8A:09:13:C2:27:EE:0B:0E:63:43:2D:88:8F

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Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
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& by email 15.01.2019

Date 18.01.2019

Reference	Application No./Patent No.
198 441 u6/ne OPPO 02	11001632.6 - 1112 / 2359834
Applicant/Proprietor: Alexion Pharmaceuticals, Inc.	

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☒ **Registered letter**

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Page 8089

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ASSOCIATION NO. 811

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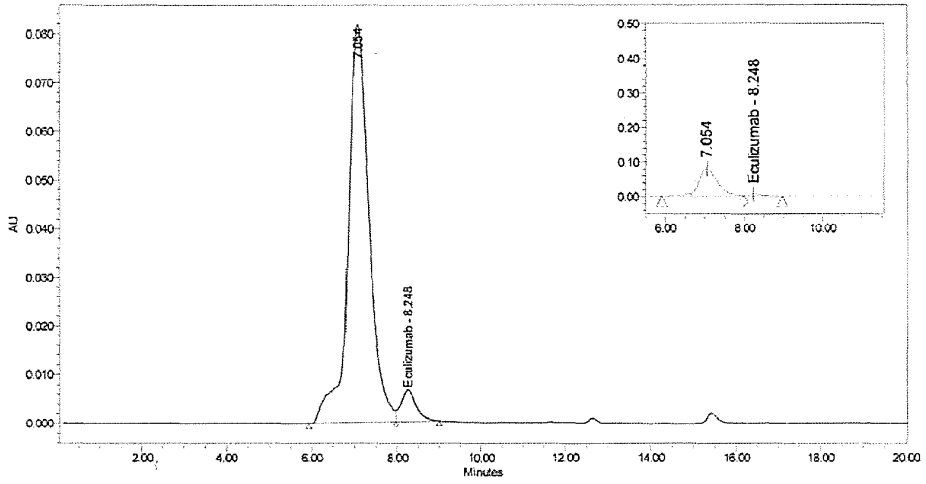
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Samsung Bioepis Exhibit No. 1002
Page 8092

Figure 1: Size Exclusion Chromatography of isolated dimer

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Page 8093

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& by email 15.01.2019

Date
18.01.2019

Reference	Application No./Patent No.
AMG17203OP OPPO 01	11001632.6 - 1112 / 2359834
Applicant/Proprietor	
Alexion Pharmaceuticals, Inc.	

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Subject:
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- ☐ Our telephone conversation of
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- ☒ Oral proceedings on 21.01.2019

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Bayerstrasse 34
(entrance via Zollstrasse 3)
80335 Munich
Germany

14 January 2019

URGENT ORAL PROCEEDINGS 21 JANUARY 2019

Dear Sirs

Opposition to European Patent No. 2359834
(Application No. 11001632.6)
ALEXION PHARMACEUTICALS, INC.
Our Ref: C411599EP PXT/ALS/hdb

I write with reference to the oral proceedings scheduled for 21 January 2019 and request admission of a new document (referred to herein as D54) into the proceedings. This letter and the enclosed document are also being sent directly to the two Opponents.

D54 is a second declaration of Dr Miano which provides the results of an assay to determine the potency of aggregated eculizumab. This declaration is filed in direct response to Opponent 1's submission of 21 November 2018 which stated in paragraph 18 that:

> *"there is no evidence of a correlation between aggregation and loss-of-activity for this specific antibody!".*

It is the proprietor's position that the skilled person in the art would generally be aware that aggregation is detrimental to the activity of biologic drugs. For instance, aggregation can affect the availability of binding sites. It is therefore generally accepted in the art that aggregation should be avoided. Eculizumab is no exception to this general principle.

However, in case there is any doubt over this matter, Dr Miano reports in D54 the result of a study into the potency of aggregated eculizumab. This confirms that the aggregated form has 50% of the C5-binding activity of eculizumab itself.

We appreciate that this new document is being filed after expiry of the Rule 116 deadline. However, we request the Opposition Division's discretion in admitting this document into proceedings.

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Samsung Bioepis Exhibit No. 1002
Page 8095

In particular, this document directly responds to a point raised in the Rule 116 submission of Opponent 1. Dr Miano's report provides a straight-forward and clear demonstration of the relative potency of the aggregate and eculizumab. It is therefore prima facie relevant to the question raised by the Opponent, namely the relevance of aggregation to eculizumab activity.

Yours faithfully

Electronically Signed
AMANDA L SIMONS
ASSOCIATION NO. 811

Samsung Bioepis Exhibit No. 1002
Page 8096

IN THE MATTER OF
European Patent No. EP 2 359 834

SECOND DECLARATION OF DR. DINO MIANO

I, Dr. Dino Miano, hereby declare as follows:

1. I am Dr. Dino Miano of Alexion Pharmaceuticals, Inc., 100 College St, New Haven, Connecticut, USA. I am the same Dr. Dino Miano that submitted a declaration known as D33 in these proceedings. Details of my background and my CV were provided with that earlier declaration.

2. I understand that one of the Opponents in this opposition has indicated that no correlation has been shown between aggregation and loss of activity for eculizumab. I was therefore asked to comment on the activity of the aggregate (dimer) form of eculizumab.

3. In 2005 we carried out a number of analyses on the eculizumab dimer sample isolated from eculizumab drug product lot 5000-001A according to according to DVL-05-100-00, "Isolation of Eculizumab Dimer and Monomer." Dimer isolation was performed by manual collection of peaks eluting from a size exclusion column. Collected peaks were pooled and concentrated with a Centriplus YM-10 centrifugal filter device.

4. These analyses included a measure of the C5 binding assay of the dimer. The C5 binding assay determines potency by measuring the ability of eculizumab samples to bind to C5 proteins. The method used for the C5 binding assay was the BioVeris Detection Analysis of C5 binding to eculizumab (Alexion ref BTM-0018.01). The assay uses an electrochemiluminescent (ECLM) reaction and photon detection technology (measured at λ = 620 nm) to determine potency of eculizumab by testing the ability of an eculizumab sample to bind C5 proteins. A ruthenium ion, necessary for the ECLM reaction, is conjugated to C5, a human complement protein. The C5 protein and eculizumab sample are incubated in a microtiter plate. rProtein-L, a biotinylated protein, is added and incubated with the C5/eculizumab complex. After the incubation, magnetized streptavidin beads are added to the plate. The antibody complex attaches to the beads through a streptavidin-biotin association. After incubation with the beads, the sample plate is measured for ECLM detection with a BioVeris system.

5. The purity of drug product lot 5000-001A, as determined by size exclusion chromatography (SEC), was 98.8% monomer and 1.2% aggregate at release (T=0, 01JUN01). This lot was stored at 2-8°C for 52 months for use in this study. The purity after 52 months was determined to be 97.25% monomer and 2.75% aggregate. The aggregate was isolated as described in paragraph 3 above.

6. The aggregate (dimer) peak isolated from drug product lot 5000-001A contained

approximately 95% dimer and 5% monomer when initially tested by SEC; refer to Figure 1.

7. The C5 binding assay was carried out according to the method described in paragraph 4 above for both eculizumab drug product lot 5000-001A and the isolated dimer sample. The source material, drug product lot 5000-001A, had a C5 binding capacity of 92.6%. The isolated dimer sample had roughly half the potency of lot 5000-001A, with a C5 binding capacity of 45.3%. A placebo (lot 2000-001A) was also tested and was below the limit of quantitation in this assay.

8. These results demonstrate that the isolated dimer has approximately 50% of the C5 binding activity of the drug product, indicating a significant drop in potency on aggregation to the dimer form.

I believe and do hereby declare that the facts stated above are to the best of my knowledge true.

Dr. Dino Miano

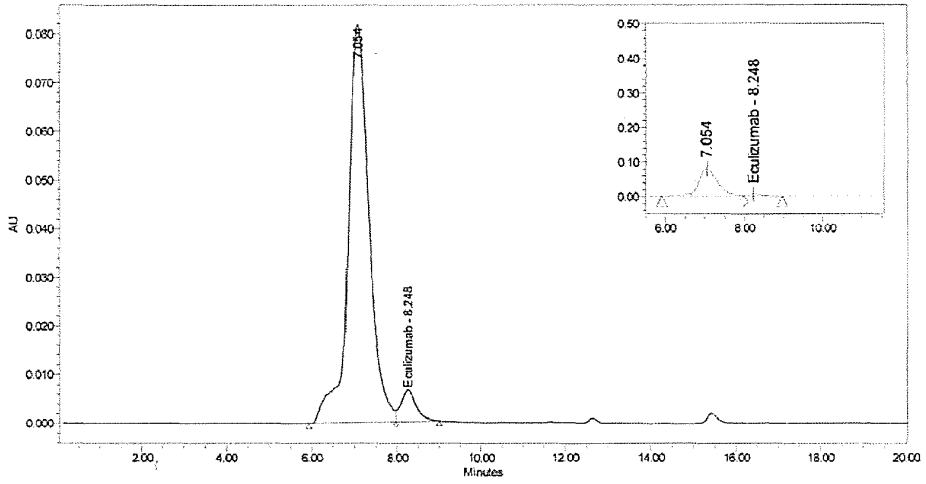
14 Jan 2019
Date

Samsung Bioepis Exhibit No. 1002
Page 8098

Figure 1: Size Exclusion Chromatography of isolated dimer

SAMPLE INFORMATION

Sample Name:	Concentrated Dimer (26JUL05)	Acquired By:	tineg
Sample Type:	Unknown	Date Acquired:	7/27/2005 11:47:18 AM
Vial:	2	Acq. Method Set:	Eculizumab SEC
Injection #:	1	Date Processed:	7/27/2005 1:53:16 PM
Injection Volume:	43.00 ul	Processing Method	Eculizumab SEC
Run Time:	20.0 Minutes	Channel Name:	Extract 214nm
Sample Set Name:	27JUL05GT01	Label:	



Peak Result Information

	Peak Name	RT(min)	Area (uV*sec)	Height (uV)	Int Type	% Area	Tailing @ 5%	Plate Count	USP Resolution
1		7.054	2795159	61667	BV	94.43	0.882	1179	
2	Eculizumab	8.248	164763	6579	VB	5.57		2358	1.513

Samsung Bioepis Exhibit No. 1002
Page 8099

Europäisches Patentamt
European Patent Office
Office européen des brevets

Application No.

11001632.6

INFORMATION

The oral proceedings of: 21.01.19

Resulted in:

☒ The European patent is revoked because at least one ground for opposition prejudices the maintenance of the European patent (Art. 101(2) EPC)

☐ The European patent is revoked because, account being taken of the amendments made by the patent proprietor during opposition proceedings, the patent and the invention to which it relates were found not to meet the requirements of the EPC (Art. 101(3)(b) EPC).

☐ The opposition(s) is/are rejected (Art. 101(2) EPC).

☐ Account being taken of the amendments made by the patent proprietor during the opposition proceedings, the patent and the invention to which it relates are found to meet the requirements of European Patent Convention (Art. 101(3)(a) EPC).

☐ Amendments filed during oral proceedings are annexed.

☐ Other:

21.01.19
Date

Signature

NB: this form is provided for the sake of information only. The written decision prevails.

EPA/EPO/OEB Form 2341 07.16

Samsung Bioepis Exhibit No. 1002
Page 8100

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Deadline:		
2	Received	
Inbox	14. Feb. 2019	Data admin.
Archive ✓	dfmp	Department KS

Application No. / Patent No.	Ref.	Date
11 001 632.6 - 1112 / 2 359 834 / 01	AMG17203OP	13.02.2019
Proprietor Alexion Pharmaceuticals, Inc.		

Provision of a copy of the minutes in accordance with Rule 124(4) EPC

The attached copy of the minutes of the oral proceedings is sent to you in accordance with Rule 124(4) EPC.

Europäisches Patentamt • European Patent Office • Office européen des brevets

Maier, Dagmar
Formalities Officer
Tel. No.: +49 89 2399 - 2366

Enclosure(s): Copy of the minutes (Form 2309)

Registered Letter
EPO Form 2042 12.07TRI

Samsung Bioepis Exhibit No. 1002
Page 8101

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Application No.: 11 001 632.6

Patent No.: EP-B-2 359 834

Minutes of the oral proceedings before the OPPOSITION DIVISION

The proceedings were public.

Proceedings opened on 21.01.2019 at 09:00 hours

Present as members of the opposition division:

Chairman:	Härtinger, Stefan
1st member:	Trifilieff-Riolo, S
2nd member:	Durrenberger, Anne
Minute writer:	Durrenberger, Anne

Present as or for the party or parties:

- For the Proprietor(s): Alexion Pharmaceuticals, Inc.

  Amanda Simon (JA Kemp)
  Pamela Tuxworth (JA Kemp)
  Todd Spalding (Alexion)
  Tara Rahemba (Alexion)
  Dino Miano (Alexion)

- For the Opponent 1: Amgen Inc.

  Ulrich Dörries (DFMP)
  Simon Geiger (DFMP)
  Tom Wrona (Amgen)

- For the Opponent 2: Hoffmann Eitle

  Leo Polz (Hoffmann Eitle)

The identity of the person/s (as well as, if applicable, that of the witness or witnesses) and, where necessary, the authorisation to represent/authority to act were checked.

Essentials of the oral proceedings and relevant statements of the parties:

9:00 Opening

1 The parties confirmed their request on file. The opponents additionally requested not to admit D33 (O2) nor D54 (O1, O2) into the proceedings as being late filed or not prima facie relevant.

MAIN REQUEST

Admissibility

2 O1 argued that D54, being available already in 2005, could and should have been filed earlier. Furthermore, D54 addressing only a minor part of the stability issue, i.e. a low decrease in aggregation not shown to have any effect of the activity of the molecule, it should not be admitted.

3 O2 added that D54 was not more relevant than what was already on file, and pertained to a totally new invention neither disclosed in the application as filed nor derivable from the experiment. In view of the availability of these data at filing date, it was clearly not the intention of the inventor to solve a problem of stability with the present invention. This applied also to D33.

4 P argued that D33 was filed in response to the notices of opposition, and D54 in response to allegations from the opponents that the skilled person would not be aware that aggregation was an issue. According to P, these data should not have been needed at all since the fact that aggregation was to be avoided was well-known, in particular that for treating PNH it is required to have as few aggregation as possible for the agent to be active.

9:25-9:30 Break

5 The chairman announced the decision of the opposition division to admit D33 and D54 into the proceedings.

Art. 100 b, c EPC

6 Regarding the right to priority and Art. 83 EPC, O1 referred to his arguments in writing, and reiterated his arguments regarding Art. 123(2) EPC. O1 insisted that the claimed invention was not sufficiently disclosed in view of P's own saying that a significant research program would have been needed to

arrive at a successful, stable eculizumab formulation, admitting the difficulties to formulate Soliris®, while the general statements in §63 of the patent did not allow to arrive at the commercial product used in D33.

7 O2 agreed with the arguments put forward by O1. He cited T63/06 for arguing that in the present, weak plausible case the burden of proof was on the patentee, insisting that the patent itself would not arrive at the formulation of Soliris®. According to O1, the legal question was not the plausibility but the undue burden.

8 P did not comment further on the priority issue. Concerning Art. 123(2) EPC, he pointed to pp. 5, 6 and 22 as a basis. Concerning Art. 83 EPC, P pointed to the absence of serious doubts substantiated by verifiable facts that the claimed formulation could not have been made. The claim being a medical use claim, it was made plausible that the claimed composition was useful in the claimed treatment of PNH. This was further supported by D25 wherein several eculizumab formulations where shown to be useful in PNH treatment.

10:20-10:40 Break

9 The chairman announced the decision of the opposition division that it considered the requirements of Art. 123(2) and 83 EPC as met, while the priority was not valid.

Art. 56 EPC

10 D1 being the closest prior art, O1 formulated the technical problem as "to provide a formulation useful in the treatment of PNH", the solution being a concentration of eculizumab at 10 mg/ml which was obvious from the range of 1 to 30 mg/ml cited in D1, as well as from D21 (4.3) and D22 (Fig. 10). The solution resulted from routine testing, while the relation between the concentration and the aggregation tendency was well-known in the art (see D20). D33 and D54 could thus be ignored.

11 With the same closest prior art, O2 formulated the technical problem as "to provide a reasonable pharmaceutical formulation for PNH", the solution of a concentration of 10 mg/ml eculizumab being an arbitrary selection, hence obvious. O2 insisted that the technical effect of the stability was not in the application as filed, in particular as no such effect could have been seen

within the short 6 month administration period of the experiment. D33 thus concerned another, distinct invention, and could not be taken into consideration.

12 P pointed to an additional difference of the claimed invention over the closest prior art, namely the eculizumab protein being distinct: D1 referred to D13 and D17, which described an antibody isotype distinct from SEQ ID NO: 2 and 4 of the patent. There were thus two effects, namely the provision of an alternative antibody (evidenced in the example of the patent), in a dosage form convenient and stable (shown in D33, D54, and further achieved with a small volume of 300 ml). The technical problem was then "to provide an alternative antibody, with an alternative formulation being stable and convenient to use", this new problem being in agreement with the requirements of the Guidelines.

Concerning the breadth of the claim and the question wether the problem was solved on the whole range, P pointed to D25 which made clear that other stable formulations could be produced, the lowest concentrations showing more stability.

The effect being shown in D33 and D54, and the new problem still pertaining to the treatment of PNH, a reformulation of the problem was to be considered acceptable.

The solution of the IgG2/4 antibody was not an obvious alternative: the skilled person had no motivation to replace the IgG4 antibody isotype of D1, while the IgG2/4 isotype of D14 was used in another context than the treatment of PNH.

In addition, while D1 did not deal with a commercially viable formulation in terms of stability, D20 taught that every antibody behaved differently. P further cited D35 and D39, arguing that the skilled person did not know if a viable antibody formulation would be obtained successfully in liquid form.

A combination of D1 with D22 would not, according to P, have been envisaged by the skilled person since D22 pertained to a pulmonary use, via nebulisation, and did not point to a 10 mg/ml liquid solution.

Finally, a combination with D29 would not add anything to the common knowledge.

13 O1 pointed to §54 of the patent, referencing D17 as well for defining eculizumab, and to the experiment which did not indicate which eculizumab form had been used.

14 O2 added that the original disclosure of the patent aimed at including all the forms of eculizumab, including fragments thereof, as equivalents without technical differences. O2 argued that this new late argument was not admissible.

12:35-12:45 Break

15 The chairman asked P whether any isotype was envisaged in the patent, to which P replied that the eculizumab antibody of the claim was the one for which the sequence is provided in the patent.

16 O1 argued that claim 1 had to be interpreted in its broadest meaning. In addition, a different eculizumab antibody would not necessarily make a technical difference, and there were no evidences that the experiments in D33 had been carried out with this specific IgG2/4 isotype.

According to O1, even in view of D25, it was not credible that the problem was solved over the whole breadth, as the formulations of D25 were limited to specific buffers and pH, while the 10 mg/ml was not even tested.

17 O2 added that P's interpretation of the claim was misleading: the claim did not require any stability, and parameters defining the latter could not even be added to the claim since there were none in the application as filed.

13:10-14:00 Break

18 The chairman asked P to what extent did the long term stability apply over the whole scope of the claim.

19 P replied an excellent stability was required from the day of manufacture until the administration, whatever the duration of storage. It was not sufficient to produce a drug without long-time stability, which was the case in D1 (clinical trial). D23 taught that a change in stability was seen even over short periods of time.

20 O1 and O2 argued that the patent did not deal with long-time treatment, nor was the claim limited to a commercial product with long-term stability. It would nevertheless be possible to treat a PNH patient over 6 month with a solution stable enough for a short period of time.

14:10-14:15 Break

21 The chairman announced that the main request did not meet the requirements of Art. 56 EPC.

14:20-14:30 Break

22 P requested the permission to introduce auxiliary requests 1a and 1b (AR1a, AR1b).

The amendment of a pH of 7.0 in AR1a found basis on p. 24, line 11, and resulted from the objection that the data provided in the proceedings could not be extended across the whole scope of the claim, while the formulations of the patent and of D25 had a pH of 7.0.

The amendment introduced in claim 1 of AR1b found basis on p. 5, lines 32-33 and p. 44

14:40-14:50 Break

AUXILIARY REQUEST 1

23 O1 and O2 referred to their previous arguments under Art. 56 EPC. O1 further raised doubts as to the compliance to Rule 80 EPC.

24 P argued that AR1 was filed in response to the combination of certain documents by the opponents.

25 The chairman announced that AR1 was not considered to meet the requirements of Art. 56 EPC.

AUXILIARY REQUESTS 1a and 1b

26 O1 argued that the requests were not converging.

He requested AR1a and b not to be admitted into the proceedings as they were filed too late with no valid reason. Concerning AR1a, the issue of the pH had already been discussed in detail. It was not prima facie apparent that Art. 123(2) EPC was met as it was arbitrarily connected to other features of the claim. Finally it did not solved the problem because many other factors were involved in stability, and it did not render the composition superior in stability.

Concerning AR1b, O2 argued that it resulted from a new argument brought up by P today, thus it was unacceptable for the parties to go through all the documents and check the sequences. O2 furthermore noted that SEQ ID NO: 4 mistakenly included the signal sequence and thus major aggregation problems were to be expected.

27 O2 also requested AR1a and b not to be admitted into the proceedings since it was filed too late and resulted from an arbitrary selection going against procedural economy. Concerning AR1a, the pH was isolated from a list of unrelated features on p. 24 of the patent. In addition, it did not solve the problem but on the contrary, a new problem would need to be formulated, requesting adjournment of the proceedings to provide new data.

Concerning AR1b, O2 raised doubts as to compliance with Art. 123(2) and (3) EPC because it was not evident from the original application what was meant under the term eculizumab; the present molecule was the result of an arbitrary selection.

Finally, O2 noted as well that the requests were not converging.

28 P argued that these requests were filed in accordance with the guidelines, E.III.8, and in response to grounds of opposition. In particular, the pH issue was discussed today only. In AR1a, the choice of pH7.0 was not arbitrary, it was an allowable single amendment which solved the same problem of stability. The claims had thus been limited to the evidences provided to solve the technical problem. AR1b was also filed in response to arguments from the opponents and provided with a new alternative to solve the problem. P pointed to the guidelines, E.VI.2, that new arguments could not be disregarded.

15:15-15:20 Break

29 The chairman announced the decision of the opposition division not to admit AR1a and AR1b into the proceedings.

AUXILIARY REQUEST 2

30 O1 stated that the earlier arguments all applied. In addition, he raised objections under Art. 83, 123(2)(3) EPC in view of the fact that the patent as granted did not contain any reference to the feature "5mg/ml" anymore.

31 O2 raised objections under Art. 84 EPC as to the scope of the claim category, which now included a process step of dilution; under Rule 80 EPC; under Art. 123(2) and (3) EPC.

32 P replied that the amendment met Art. 123(2) EPC since it was based on the application as filed. The loss of the passage in the description relating to this step was due to a printing error. The requirements of Art. 84 EPC were met as well: the new feature defined further the medical use. Concerning Art. 56 EPC, the skilled person could, but would not have carried out this dilution to 5 mg/ml.

33 According to O1, the could/would approach failed because the novel feature did not result in a new technical effect. O1 insisted that the Druckexemplar had been accepted, hence the deleted paragraph could not be considered as a printing error.

34 According to O2, a reasonable discussion on inventive step was not possible as no technical effect was associated to this novel feature.

15:35-15:45 Break

35 The chairman announced the decision of the opposition division that AR2 did not meet the requirements of the EPC. The patent was revoked.

15:50 Closing of the proceedings

After deliberation of the opposition division,

- the chairman announced the following **decision** :

"The European patent is revoked."

Regarding the reasons for the decision, the chairman referred to:

Article 101(2) EPC, first sentence: the following ground(s) for opposition mentioned in Article 100 EPC prejudice(s) the maintenance of the patent as granted.

The chairman **closed the oral proceedings** on 21.01.2019 at 15:50 hours.

signed:

Härtinger, Stefan
. . . . . .
Chairman

signed:

Durrenberger, Anne
. . . . . .
Minute Writer

Enclosure(s):
Auxiliary requests 1A and 1B

Samsung Bioepis Exhibit No. 1002
Page 8110

Auxiliary Request 1A

Claims

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, having a pH of 7.0.

Patentansprüche

1. Pharmazeutische Zusammensetzung zur Verwendung in der Behandlung eines Patienten, der an paroxysmaler nächtlicher Hämoglobinurie (PNH) leidet, wobei die Zusammensetzung eine 300-mg-Eculizumab-Einmal-Darreichungsform ist, umfassend 30 ml einer 10 mg Eculizumab pro ml enthaltenden sterilen, konservierungsmittelfreien Lösung.

Revendications

1. Composition pharmaceutique pour une utilisation dans le traitement d'un patient atteint d'une hémoglobinurie paroxystique nocturne (HPN), dans laquelle la composition est une forme galénique à usage unique de 300 mg d'eculizumab comprenant 30 ml d'une solution à 10 mg d'eculizumab/ml, stérile et sans conservateur.

21 Jan 2019

Samsung Bioepis Exhibit No. 1002
Page 8111

Auxiliary Request 1B

Claims

1. A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution, wherein eculizumab comprises a heavy chain consisting of SEQ ID NO: 2 and a light chain consisting of SEQ ID NO: 4.

Patentansprüche

1. Pharmazeutische Zusammensetzung zur Verwendung in der Behandlung eines Patienten, der an paroxysmaler nächtlicher Hämoglobinurie (PNH) leidet, wobei die Zusammensetzung eine 300-mg-Eculizumab-Einmal-Darreichungsform ist, umfassend 30 ml einer 10 mg Eculizumab pro ml enthaltenden sterilen, konservierungsmittelfreien Lösung.

Revendications

1. Composition pharmaceutique pour une utilisation dans le traitement d'un patient atteint d'une hémoglobinurie paroxystique nocturne (HPN), dans laquelle la composition est une forme galénique à usage unique de 300 mg d'eculizumab comprenant 30 ml d'une solution à 10 mg d'eculizumab/ml, stérile et sans conservateur.

A Smith
21 Jan 2019

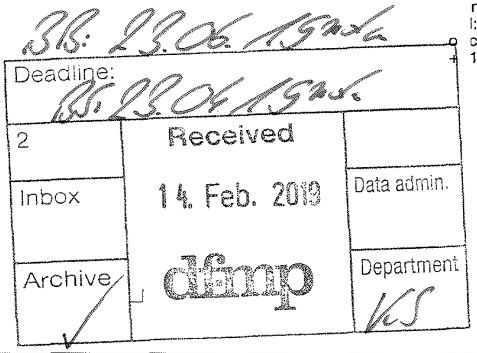
Samsung Bioepis Exhibit No. 1002
Page 8112

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Application No. / Patent No.	Ref.	Date
11 001 632.6 - 1112 / 2 359 834 / 01	AMG17203OP	13.02.2019
Proprietor Alexion Pharmaceuticals, Inc.		

Decision revoking the European Patent (Art. 101(2) and 101(3)(b) EPC)

The Opposition Division - at the oral proceedings dated 21.01.2019 - has decided:

European Patent No. EP-B- 2 359 834 is revoked.

The reasons for the decision are enclosed.

Possibility of appeal
This decision is open to appeal. Attention is drawn to the attached text of Articles 106 to 108 and Rules 97 to 98 EPC.

Opposition Division:

Registered letter with advice of delivery
EPO Form 2331 12.07TRI

Samsung Bioepis Exhibit No. 1002
Page 8113

Chairman: Härtinger, Stefan
2nd Examiner: Durrenberger, Anne
1st Examiner: Trifilieff-Riolo, S

Maier, Dagmar
Formalities Officer
Tel. No.: +49 89 2399-2366

Enclosure(s): 15 page(s) reasons for the decision (Form 2916)
Wording of Articles 106 - 108 and Rules 97-98 EPC (Form 2019)
Minutes of oral proceedings

to EPO postal service: 06.02.19

Registered letter with advice of delivery
EPO Form 2331 12.07TRI

Samsung Bioepis Exhibit No. 1002
Page 8114

I. Summary of Facts and Submissions

1. European patent 2 359 834 having the title "Treatment of paroxysmal nocturnal hemoglobinuria patients by an inhibitor of complement" is based upon European patent application No. 11 001 632.6 filed on 15-03-2007 . It claims priority of US 783070 filed on 15-03-2006.

The mention of the grant of the patent has been published in the European Patent Bulletin of 09-11-2016.

Proprietor of the patent (hereinafter P) is
Alexion Pharmaceuticals, Inc.
100 College Street
New Haven, CT 06510
US.

2. Notices of opposition have been filed by
Opponent 1 (hereinafter O1):
Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320-1799
US
on 04-08-2017

Opponent 2 (hereinafter O2):
Hoffmann Eitle
Patent- und Rechtsanwälte PartmbB
Arabellastrasse 30
81925 München
DE
on 09-08-2017.

The opponents requested revocation of the patent in its entirety as follows:

O1 contended that the subject-matter extends beyond the content of the application as filed (A. 100(c) ; A. 76(1) and A. 123(2) EPC) and that the invention is not sufficiently disclosed (A. 100(b); A. 83 EPC).

O1 and O2 disputed the validity of the claimed priority (A. 87 EPC) in relation to the determination of the proper prior art documents.

O1 and O2 both contended that the claimed subject-matter does not meet the requirements for inventive step (A. 100(a), A. 56 EPC).

Both O1 and O2 made subsidiary requests for oral proceedings.

D1 to D32 have been submitted within the opposition period (see §11 hereafter).

3. On 30-10-2017 P requested an extension of two months to respond to the notices of opposition.

P's request was granted on 09-11-2017.

4. In a letter received on 22-03-2018, P requested the rejection of the oppositions (Article 101(2) EPC) and maintenance of the patent as granted. P made a subsidiary request for oral proceedings.

P filed D33 to D38 in support of his argumentation.

5. On 05-07-2018, the opposition division (hereinafter OD) issued a summons to attend oral proceedings on 21-01-2019.

6. On 19-11-2018, O1 requested that Dr. Treuheit (author of D23) be allowed to speak at the oral proceedings.

On 21-11-2018, O1 reacted to the OD's summons and filed D42 to D49.

7. On 20-11-2018, O2 requested not to admit D33 into the proceedings, reacted to the OD's summons and filed D50 to D53.

8. On 21-11-2018, P filed two auxiliary requests (AR1 and AR2), D39 to D41 and requested that Dr. Miano (author of D33) be allowed to speak at the oral proceedings.

9. On 14-01-2019, P filed a further document D54 and requested to admit it into the proceedings.

10. On 21-01-2019, oral proceedings were held in the presence of all parties.

At the beginning of the oral proceedings O1 and O2 additionally requested not to admit D54 into the proceedings.

11. The numbering of the documents is that filed by P on 14-12-2018 to which document D54 is added.

12. The decision is based on the following requests:

Patent as granted (main request).

Auxiliary requests 1 (AR1) and 2 (AR2) filed on 21-11-2018.

Auxiliary requests 1A (AR1A) and 1B (AR1B) filed during oral proceedings.

II Grounds for the decision:

1. The oppositions meet the requirements of A. 99(1) and 100 EPC and Rules 3(1), 76(1) and 76(2) EPC. They are thus admissible.

2. Admissibility of documents D33 and D54:

2.1. O1 and O2 object that D54 is late filed and could have been filed earlier since it refers to analyses made in 2005 (p. 1, point 3.). O1 further argues that the purpose of D54 which is to show that aggregation of eculizumab correlates with a loss of its activity is not reached, because D54 would only relate to a very specific formulation of eculizumab and because aggregation might be reversible.

P answers that D54 has been filed in response to O1's comment according to which P had not shown that aggregation of eculizumab led to a loss of its activity. In P's view, this was part of the general knowledge of the skilled person and hence did not need to be demonstrated.

The OD considers that D54 has indeed been filed as a direct response to O1's comments above and decides to admit it into the proceedings.

2.2. O2 contends that D33 should not be admitted into the proceedings because it is a post-published document which attempts to demonstrate that the eculizumab composition of claim 1 shows an unexpected effect which was neither present nor deducible from the application as originally filed.

P replies that D33 has been filed as soon as possible, that is within the prescribed period of R. 116 EPC, as a response to the objections for lack of inventive step raised in the notices of opposition.

The OD concurs with P's view and decides to admit D33 into the proceedings.

2. MAIN REQUEST:

It is the granted patent which contains a unique claim reading as follows:

"A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ ml sterile, preservative free solution."

2.1. Validity of the claimed priority (A. 87 EPC):

Similarly to the opponents, the OD notes that the priority document US 783070 does not disclose a single-use dosage form of a liquid and sterile composition comprising 30 ml of a 10 mg eculizumab/ml and no preservative, let alone its use to treat PNH.

P does not dispute this.

Thus the priority is not valid and the actual filing date of the present patent is the 15-03-2007.

2.2. Extension of the subject-matter of the patent in suit beyond the content of the application as originally filed (A. 123(2); A. 76(1) EPC):

According to O1 the parent application as originally filed would only teach the subject-matter of granted claim 1 in relation to the fact that the dosage form is a single **unit** dosage form. In O1's view this would mean that the 300 mg of eculizumab should be administered as a single dose. Since granted claim 1 does not contain such a feature, the subject-matter of the claim would not be clearly and unambiguously derivable from the application as filed and would thus extend beyond it.

P replies that the wording of granted claim 1 concerning the expression "300 mg eculizumab single-**use** dosage form comprising 30 ml of a 10 mg eculizumab/ml sterile, preservative free solution" is found *expressis verbis* in the parent application as originally filed on p. 5, l. 8-9, p. 6, l. 22-23 and p. 22, l. 12-13.

The OD agrees with P and further observes that in these passages, there is no mandatory link between "a single-**use** dosage form" and "a single-**unit** dosage form". Both are cited as separate possible embodiments so that O1's reasoning does not apply.

Hence the OD concludes that the requirements of A. 123(2) and A. 76(1) EPC are met.

2.3. Sufficiency of disclosure (A. 83 EPC):

O1 objects on one hand that the skilled person would not be able to determine the correct structure of eculizumab and on another hand that he would not be able to produce a formulation such as that used in claim 1.

2.3.1. Determination of the structure of eculizumab:

O1 notes that, while eculizumab is described in different documents of the prior art (D1 to D5, D11 to D15), the description of the patent offers divergent definitions for it and particularly does not provide a correct sequence of its light chain. Particularly, the light chain of eculizumab (SEQ ID NO: 4) depicted in the patent would comprise 22 additional amino acids more than the light chain of eculizumab described in the post published D25 (§94). In O1's view this would confuse the skilled person and could even lead to conclude that the antibody described in the patent is not eculizumab.

P retorts that the SEQ ID NO: 4 of the patent describes the correct light chain of eculizumab in its **immature** state. This means that it contains at its N-terminus a 22-residue signal peptide starting with MDM (Met-Asp-Met)... and ending with RC(Arg-Cys) which is absent from the mature peptide. It would be part of the skilled person's general knowledge (illustrated by e.g. D38) that this 22-residue peptide would correspond to a signal peptide of human kappa light chains. Hence the skilled person would thus immediately recognize that SEQ ID NO: 4 of the patent is an **immature** light chain and would know that the mature light chain would start at amino acid 23 of this SEQ ID NO: 4.

The starting point to determine if the invention is sufficiently disclosed is the patent and not a post-published document.

In that respect, the OD observes that the patent describes the variable heavy chain and light chain segments of eculizumab (SEQ ID NO: 1 and 3) as well as its full heavy and light chains (SEQ ID NO: 2 and 4).

The skilled person would thus consider these information to provide eculizumab. O1's argument is based on a comparison of these information with a post published document. Already for this reason such an argument must fail.

Moreover, since no proof of the contrary is available, the OD can see no reason not to accept P's argument concerning the fact that the skilled person would recognize that the SEQ ID NO: 4 is the immature light chain of eculizumab and that the mature light chain would/should start at amino acid 23 of this sequence.

Hence it is the OD's view that the patent discloses enough information for a skilled person to produce an eculizumab protein.

2.3.2. Provision of the formulation used in the claim:

O1 and O2 object that the patent does not describe a single precise formulation corresponding to the one used in the claim. They note that §63 of the patent lists numerous excipients and that no specific pH is indicated. They further refer to a P's statement in his letter of 22-03-2018 (§5.5.5) according to which "a significant research program would have to be conducted to fully investigate the stability and biological integrity of the antibody, as well as the various factors (and their interplay) affecting each" and consider that it proves the soundness of their objection. In addition, the opponents refer to D21 and D40 which would emphasize that formulation of antibodies in general is a difficult matter.

P firstly notes that no evidence has been provided which would show that the formulation used in claim 1 cannot be produced. P also argues that the subject-matter of the claim is a therapeutic use and not a composition per se and that the claim does not relate to the stability of the composition. This would mean that what must be disclosed in the patent is that the composition is useful to treat PNH and this would be shown in the examples. Moreover, according to P it could be derived from §63 and 64 of the patent that the formulations are made in a usual way. D25 would also prove that different formulations can be made.

The OD agrees with P that the subject-matter of the claim is not a composition *per se* but a therapeutic use. In order to perform the invention, however, some kind of formulation of eculizumab must be provided and this formulation must be liquid, sterile, preservative free, comprise 10 mg eculizumab/ ml and have a volume of 30 ml.

§63 of the patent gives information about how to produce such a formulation: a range of pH is indicated (from about 5.0 to about 7.0), as well as specific excipients. References to several patent documents are also made.

No evidence has been provided that by using the indications given in §63 it was not possible to formulate a composition of eculizumab as described above. The fact that antibodies in general are known to be difficult to formulate is not enough to obviate the need for such a proof.

In view of the above, the OD is of the opinion that the patent contains enough information to enable the skilled person to provide a formulation as used in the claim.

The OD thus concludes that the granted patent meets the requirements of A. 83 EPC.

2.4. Inventive step (A. 56 EPC):

All parties agree that D1 represents the closest prior art and the OD sees no reason to decide otherwise.

D1 (§82) describes a study wherein PNH patients are treated with a weekly 600 mg intravenous infusion of eculizumab during 4 weeks, followed by 900 mg of eculizumab 1 week later and then 900 mg on a bi-weekly basis. The study was extended for a total of 2 years for 10 out of the 11 original patients.

The effects of this treatment on different physiological parameters and symptoms involved in PNH are analyzed in D1 and show that the treatment is successful.

2.4.1. The opponents consider that the differences between the disclosure of D1 and the subject-matter of the granted claim are: eculizumab is in a single-use dosage form of a solution comprising a total amount of 300 mg of eculizumab

the eculizumab concentration is 10 mg/ml

the volume of the dosage form is 30 ml

the solution is free of preservative.

According to the opponents, the feature concerning the sterility can be regarded as implicitly disclosed by D1 since D1 describes an intravenous infusion.

Further, no technical effect would result from any of these differences so that the problem with respect to D1 would be the provision of a pharmaceutical composition comprising eculizumab for use in the clinical study described in D1.

More specifically, the total amount of 300 mg of eculizumab would be obvious in view of D1 itself, the feature concerning the lack of preservative would be obvious in view of common general knowledge or in view of regulatory aspects (O1; D19) or mention of such a possibility in other prior art documents (O2; D29).

In O2's view, the claimed concentration of 10 mg/ml would be part of the common general knowledge in the field of proteins/ antibodies (D20, D29 both summarized in D31 and D32).

In O1's view the concentration of 10 mg/ml would be an arbitrary choice in the context of routine experimentation when considering the teaching of D5, D20, D21 and D22. This would be emphasized by D23 which depicts assays assessing the stability of eculizumab solutions at 2, 5, 10, 30 and 50 mg/ml and shows that they are all equally stable for 13 weeks at 4°C and 25°C.

It would follow from the obvious total amount (300 mg) and from the obvious concentration (10 mg/ml) that a volume of 300 ml would also be obvious.

A further argument presented by O1 and O2 is that the additional data submitted in D33 and D54 would not be linked to the claimed therapeutic effect but to the composition of eculizumab *per se* and would thus have no relevance in assessing inventive step of the claimed therapeutic use.

It is also the opponents' view that D33 relates to a technical effect which was not present in the patent which would be a further reason for regarding it as irrelevant.

Should the data of D33 nevertheless be taken into account, they would not be sufficient to demonstrate that **all** the compositions encompassed by the claim would have the stability of the (only) composition described in D33. In other words it would not have been shown that the problem of providing stable eculizumab compositions would be solved over the whole breadth of the claim.

2.4.2. According to P, there would be a further difference between the teaching of D1 and the claimed subject-matter which would be the eculizumab itself. The eculizumab antibody used in the patent is an IgG2/4 antibody which would be different from the antibody used in D1 which would be an IgG4 isotype.

Hence the problem would reside in providing an alternative antibody in a highly stable formulation in a form easy to use to treat PNH.

P considers that the skilled person would have had no reason to use a different eculizumab isotype than that used in D1 and that the other differences (particularly the 10 mg/ml concentration) would lead to a special technical effect which would be that the eculizumab solution used in the claim demonstrates the ideal balance of properties for its use to treat PNH, including enhanced maintenance of biological integrity upon prolonged storage in the absence of preservative and low-volume liquid formulation. This effect would be demonstrated in post filed D33 which compares the stabilities of a 10 mg/ml and a 30 mg/ml solution of eculizumab and shows a superior stability for the 10 mg/ml solution.

P asserts that although this effect is not mentioned in the original application, it would be intimately related to the technical problem set out in the original application which is to provide a treatment for PNH because it is mandatory for any medicament, including antibodies, to have a good stability so that the post filed data of D33 would have to be taken into account in assessing inventive step.

P also contends that D25 would prove that the problem of providing stable eculizumab compositions would be solved over the whole breadth of the claim.

2.4.3. At first sight the differences between D1 and the subject-matter of the patent are that in the latter, eculizumab is in a single-use dosage form of a solution comprising a total amount of 300 mg of eculizumab, the eculizumab concentration is 10 mg/ml, the volume of the dosage form is 30 ml and the solution is free of preservative.

The OD concurs with the opponent's view that sterility of the composition is implicit.

Concerning the P's assertion that the antibody eculizumab of the patent would be different from that of D1, the OD notes that D1 does not recite any sequence for eculizumab. D1 specifies that eculizumab is a trade name for the antibody h5G1.1 and refers to two documents, D17 and D13 which would describe its preparation and that of other functional fragments of it. It is further observed that the patent itself (§54) states that "the antibody is disclosed in US Patent 6,355,245 (i.e. D17) and is named eculizumab".

This would mean that the eculizumab of the patent and that disclosed in D17, and hence in D1, is the same antibody. In appears however that D17 (ex. 11, col. 44 (12)) does not give a straightforward/complete sequence of eculizumab.

Turning to D13 referred to in D1, Figure 1 shows the sequences of the h5G1.1 heavy and light variable regions. It is observed that the variable region of the h5G1.1 heavy chain of D13 is identical to that of the patent whereas the variable region of the h5G1.1 light chain of D13 has a different amino acid at position 38 compared to the corresponding amino acid of the variable region of the light chain of the patent (i.e. position 60 of SEQ ID NO: 3 and 4 of the patent because of the presence of the signal peptide at the N-terminus).

In view of the lack of information of D17, to which however the patent refers in §54 and of the information of D13, it cannot be determined with certainty whether the eculizumab of D1 is different from the eculizumab of the granted claim.

Although P emphasizes that the eculizumab of the granted claim is that with the sequence recited in the patent, the OD notes that the granted claim does not specify any sequence for "eculizumab" and hence is of the opinion that it must be considered that the "eculizumab" of D1 falls within the definition of "eculizumab" as claimed.

Accordingly, the differences between D1 and the granted subject-matter are those listed at the beginning of the present § 2.4.3.

It is the OD's view that the features concerning the lack of preservative and the amount of 300 mg per dosage form are already suggested in D1. §62 states that "the formulations may contain preservatives" which imply that their presence is only optional.

The amounts of eculizumab administered in D1 are 600 mg (injected during thirty minutes) or 900 mg. Hence a dosage form of 300 mg constitutes an obvious ideal amount for one dose.

D1 however fails to disclose any concentration for the eculizumab composition which is administered to the PNH patients.

This is therefore the problem to which the skilled person is confronted.

It must thus be examined whether the choice of 10 mg/ml is obvious in view of the prior art/general knowledge in the field.

The OD observes that the only document actually describing concentrations of eculizumab liquid compositions is D22. The compositions of D22 are used in a nebulizer to treat pulmonary diseases. The mechanism involved in treating these diseases is the same as that involved in the treatment of PNH, namely to inhibit activation of the complement cascade (D22, e.g. §4). Although the OD agrees with P that the manner in which a drug is to be administered is a critical factor in the formulation of the drug, it must be noted that the question to be answered in the present case is: what must be the concentration of eculizumab in a solution for said solution to be suitable to be administered to a patient. Since D22 also describes solutions, the OD is convinced that the skilled person would have considered the eculizumab concentrations described in this document, despite the fact that the solutions of D22 are nebulized and not administered intravenously. As a matter of fact, this teaching is an indication that solutions of eculizumab at these concentrations have adequate properties to be administered to a patient in terms of safety and efficiency.

Formulation 1 in Fig. 10 of D22 (in relation to §170 and 171) describes solutions where eculizumab is present at concentrations between 1 and 30 mg/ml.

Following the above reasoning, the skilled person confronted with the problem of finding suitable concentrations of eculizumab would have been taught by D22 that concentrations between 1 and 30 mg/ml of eculizumab are suitable. In particular D22 describes a liquid formulation comprising 30 mg/ml of eculizumab (§171).

Thus the skilled person would have completed the teaching of D1 by this piece of information and would have provided eculizumab solutions comprising between 1 and 30 mg/ml eculizumab.

The choice of a concentration of 10 mg/ml eculizumab represents an arbitrary choice among the quite narrow range proposed by D22 for which, at first sight, no inventive merit can be acknowledged.

If however this choice would lead to a technical effect which could not have been foreseen, the conclusion on inventive step could be different.

According to P, this would be the case because a 10 mg/ml eculizumab formulation has an unexpected improved long-term stability compared to a 30 mg/ml eculizumab formulation as demonstrated by D33.

D33 is a post-published document which the OD formally admitted into the proceedings (see point 2.2. above). This does not however mean that the data provided by D33 can automatically be taken into account when examining inventive step.

It must first be determined whether the technical effect illustrated by D33, i.e the fact that the 10 mg/ml eculizumab solution is more stable than the 30 mg/ml solution, is implied by or at least related to the technical problem initially suggested in the originally filed application (G-VII, 11) which in the present case is to improve the quality of life of PNH patients and/or to prolong their health-adjusted life expectancy (§6 to 10 of the patent).

P contends that any treatment for PNH must, to ensure efficacy, be provided in a form which maintains its biological integrity and activity during any period of storage. The OD understands that in P's view, this implies that pharmaceutical activity and stability are related in the sense that the skilled person would always contemplate the two aspects together. In the present case this would mean that even if not expressly stated in the original application, the problem of stability of the eculizumab solution would be present.

The OD disagrees.

The subject-matter of the granted claim is a therapeutic use of a solution of eculizumab to treat PNH. The subsequent data of D33 concern the stability of the solution and show that the 10 mg/ml eculizumab solution is stable for a total period of 48 months.

The OD cannot see which effect this has on the claimed therapeutic treatment of PNH. The OD agrees with P that long-term stability is an important factor as far as the **commercial** composition is concerned. This however is not part of the claimed subject-matter, which relates to the treatment of PNH.

The long-term stability of the 10 mg/ml solution has no bearing on the patient, contrary to a lower toxicity for instance (as exemplified in G-VII, 11).

Hence the OD concludes that the stability of the eculizumab formulation is not related to the claimed therapeutic use in such a sense that the problem can be reformulated by taking into account the data of D33.

The objective problem with respect to D1 thus remains as stated previously and is the determination of a concentration for the eculizumab composition which is administered to the PNH patients of D1.

As explained above, the choice of 10 mg/ml is arbitrary in view of D22 and the other features of the composition are obvious in view of D1 itself.

The OD hence concludes that the subject-matter of the granted claim does not meet the requirements for inventive step.

3. AUXILIARY REQUEST 1:

3.1. Its unique claim reads:

"A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ ml sterile, preservative free solution, wherein administration is by intravenous infusion."

3.2. It has been filed on 21-11-2018 with P's answer to the notices of opposition. The OD sees no reason to dispute its admissibility (R. 80 EPC).

3.3. The only additional feature with respect to the granted claim is the mode of administration by intravenous infusion.

Since this feature is part of the teaching of D1, the reasoning made above under point 2.4.3 applies to the subject-matter of this claim which hence does not meet the requirements for inventive step (A. 56 EPC).

Auxiliary request 1 is not acceptable.

4. AUXILIARY REQUEST 1A:

4.1. Its unique claim reads:

"A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ ml sterile, preservative free solution, having a pH of 7.0."

4.2. Admissibility (R. 80 EPC):

This matter must be settled because the request has been filed at the oral proceedings.

The only additional feature with respect to the granted claim is that the pH of the eculizumab composition has a value of 7.0.

The OD notes that this feature concerns the eculizumab formulation itself and not the claimed therapeutic use to treat PNH. Hence, notwithstanding the fact that, as stated by the opponents, D22 discloses a pH range of 6-8 for eculizumab solutions, the OD cannot *prima facie* see how such a feature would be able to overcome the objection for lack of inventive step detailed under point 2.4.3 above.

For this reason AR1A is not admitted into the proceedings.

5. AUXILIARY REQUEST 1B:

5.1. Its unique claim reads:

"A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ ml sterile, preservative free solution, wherein eculizumab comprises a heavy chain consisting of SEQ ID NO:2 and a light chain consisting of SEQ ID NO:4."

5.2. Admissibility (R. 80 EPC):

Similarly to AR1A, the request has been filed at the oral proceedings.

It is firstly noted that AR1B and AR1A are not convergent since the additional feature of AR1B is the specification of the sequences constituting eculizumab.

Further, arguments taking this feature into account have only been brought up by P on the day of the oral proceedings and have given rise to objections from the opponent's part. In particular the opponents objected that they could not have foreseen that this feature would have to be analyzed in detail and were thus not in a position to properly argue with respect to it on the very day of the oral proceedings.

On another hand, although this feature had to be analyzed at least to some extend in the present decision, the analysis proves to be complex (point 2.4.3. above).

For the reasons exposed above the OD cannot consider that AR1B is *prima facie* clearly allowable.

AR1B is thus not admitted into the proceedings.

6. AUXILIARY REQUEST 2:

6.1. Its unique claim reads:

"A pharmaceutical composition for use in treating a patient afflicted with paroxysmal nocturnal hemoglobinuria (PNH), wherein the composition is a 300 mg eculizumab single-use dosage form comprising 30 ml of a 10 mg eculizumab/ ml sterile, preservative free solution, wherein the composition is administered via IV infusion and diluted to a final concentration of 5 mg/ml prior to administration."

3.2. It has been filed on 21-11-2018 with P's answer to the notices of opposition. The OD sees no reason to dispute its admissibility (R. 80 EPC).

3.3. The additional features with respect to the granted claim are the mode of administration and the final dilution prior to administration.

The OD agrees with P that the final concentration and the mode of administration are features which pertain to the therapeutic use of eculizumab to treat PNH and that they do not render the claim unclear.

3.4. Further the OD observes that these features are described in the original application p. 22, l. 27-29 and that their presence in the claim does not extend the protection afforded by the granted patent.

The requirements of A. 123(2) and 123(3) EPC are met.

As a remark, the fact that the additional final concentration is not present any more in the description of the granted patent, whatever the cause may be, is of no relevance when examining the requirements of A. 123(2) EPC.

3.5. As far as inventive step is concerned, applying the reasoning exposed for the granted patent (point 2.4.3. above) and noting that intravenous infusion is described in D1, the additional technical feature to be taken into account is the final concentration of 5 mg/ml.

No technical effect linked to this effect is shown so that, since it has been found obvious with respect to D1 to provide an eculizumab solution in a dosage form comprising 300 mg of eculizumab with a concentration of 10 mg/ml to treat PNH patients, the problem can only be seen in finding a convenient final dilution for administering this eculizumab dosage form intravenously.

The skilled person would have arrived at such a dilution in the course of routine development when looking for an adequate mode of administering the eculizumab solution, taking into account e.g. the total amount needed by the patient, the most appropriate speed of administration...

Therefore, AR2 does not meet the requirements for inventive step (A. 56 EPC).

7. Conclusion:

Taking account of the amendments made by the patent proprietor during opposition proceedings, European patent EP 2 359 834 is revoked pursuant to A. 101(2) and 101(3)(b) EPC.

Article 106
Decisions subject to appeal

(1) An appeal shall lie from decisions of the Receiving Section, Examining Divisions, Opposition Divisions and the Legal Division. It shall have suspensive effect.

(2) A decision which does not terminate proceedings as regards one of the parties can only be appealed together with the final decision, unless the decision allows a separate appeal.

(3) The right to file an appeal against decisions relating to the apportionment or fixing of costs in opposition proceedings may be restricted in the Implementing Regulations.

Rule 97
Appeal against apportionment and fixing of costs

(1) The apportionment of costs of opposition proceedings cannot be the sole subject of an appeal.

(2) A decision fixing the amount of costs of opposition proceedings cannot be appealed unless the amount exceeds that of the fee for appeal.

Rule 98
Surrender or lapse of the patent

The decision of an Opposition Division may be appealed even if the European patent has been surrendered in all the designated Contracting States or has lapsed in all those States.

Article 107
Persons entitled to appeal and to be parties to appeal proceedings

Any party to proceedings adversely affected by a decision may appeal. Any other parties to the proceedings shall be parties to the appeal proceedings as of right.

Article 108
Time limit and form

Notice of appeal shall be filed, in accordance with the Implementing Regulations, at the European Patent Office within **two months** of notification of the decision. Notice of appeal shall not be deemed to have been filed until the fee for appeal has been paid. Within **four months** of notification of the decision, a statement setting out the grounds of appeal shall be filed in accordance with the Implementing Regulations.

Further information concerning the filing of an appeal

(a) Notice of appeal can be filed in accordance with Rule 1 and Rule 2(1) EPC, by delivery by hand, by post, or by technical means of communication. The filing has to comply with the details and conditions and, where appropriate, any special formal or technical requirements laid down by the President of the European Patent Office (R. 99(3) EPC).

(b) The addresses of the filing offices of the European Patent Office are as follows:

(i) European Patent Office
D-80298 Munich
Germany

Fax: +49 89 2399-4465

(ii) European Patent Office
Postbus 5818
NL-2280 HV Rijswijk (ZH)
The Netherlands

Fax: +31 70 340-3016

(iii) European Patent Office
D-10958 Berlin
Germany

Fax: +49 30 259 01-840

(c) The notice of appeal must contain the name and address of the appellant in accordance with the provisions of Rule 41(2)(c) EPC, an indication of the decision impugned, and a request defining the subject of the appeal. In the statement of grounds of appeal the appellant shall indicate the reasons for setting aside the decision impugned, or the extent to which it is to be amended, and the facts and evidence on which the appeal is based (R. 99(1) and (2) EPC). The notice of appeal and any subsequent submissions stating the grounds for appeal must be signed (R. 50(3) EPC).

(d) The fee for appeal is laid down in the Rules relating to Fees. The schedule of fees and expenses of the EPO or a reference to the current version is regularly published in the Official Journal of the European Patent Office under the heading "Guidance for the payment of fees, expenses and prices". Fee information is also published on the EPO website under www.epo.org/fees.

17581 U.S. PTO 051105

PTO/SB/05 (04-05)
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UTILITY PATENT APPLICATION TRANSMITTAL

(ONLY FOR NEW NONPROVISIONAL APPLICATIONS UNDER 37 CFR 1.53(B))

Attorney Docket No.	ALEX-P01-001
First Inventor	Yi Wang
Title	NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES
Express Mail Label No.	EV543607732US

113243 U.S.PTO 11/127438 051105

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

1. [X] **Fee Transmittal Form** (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. [ ] **Applicant claims small entity status.** See 37 CFR 1.27.
3. [X] **Specification** [*Total Pages* 46]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. [X] **Drawing(s)** *(35 U.S.C. 113)* [*Total Sheets* 16]
5. **Oath or Declaration** [*Total Sheets*]
   a. [ ] Newly executed (original or copy)
   b. [ ] A copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 18 completed)*
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. [X] **Application Data Sheet.** See 37 CFR 1.76
7. [ ] **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   [ ] Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission** *(if applicable, items a. – c. are required)*
   a. [ ] Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or ii. [ ] Paper
   c. [ ] Statements verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

9. [ ] **Assignment Papers** (cover sheet & document(s))
   Name of Assignee:
10. [ ] **37 CFR 3.73(b) Statement** *(when there is an assignee)* [ ] **Power of Attorney**
11. [ ] **English Translation Document** *(if applicable)*
12. [ ] **Information Disclosure Statement** (PTO/SB/08 or PTO-1449)
    [ ] Copies of citations attached
13. [ ] **Preliminary Amendment**
14. [X] **Return Receipt Postcard** (MPEP 503) *(Should be specifically itemized)*
15. [ ] **Certified Copy of Priority Document(s)** *(if foreign priority is claimed)*
16. [ ] **Nonpublication Request** under 35 U.S.C.122 (b)(2)(B)(i). Applicant must attach form PTO/SB/35 or its equivalent.
17. [ ] Other:

18. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in the first sentence of the specification following the title, or in an Application Data Sheet under 37 CFR 1.76:*

[ ] Continuation [ ] Divisional [X] Continuation-in-part (CIP) of prior application No.: 10/655861

Prior application information: Examiner Not Yet Assigned *Art Unit:* N/A

19. CORRESPONDENCE ADDRESS

[X] The address associated with Customer Number: 28120 *OR* [ ] Correspondence address below

Name	Anita Varma ROPES & GRAY LLP				
Address	One International Place				
City	Boston	State	MA	Zip Code	02110-2624
Country	US	Telephone	(617) 951-7000	Email	

Signature	Anita Varma	Date	May 11, 2005
Name (Print/Type)	Anita Varma	Registration No. (Attorney/Agent)	43,221

I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV543607732US, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: 5-11-05 Signature: Maura A. Gallagher (Maura A. Gallagher)

AMG1049

Samsung Bioepis Exhibit No. 1002
Page 8132

17581 U.S. PTO
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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 1,000.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Examiner Name	Not Yet Assigned
Art Unit	N/A
Attorney Docket No.	ALEX-P01-001

METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☒ Deposit Account Deposit Account Number: 18-1945 Deposit Account Name: Ropes & Gray LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☒ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
Utility	300	150	500	250	200	100	1,000.00
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	**Fee ($)**	**Small Entity Fee ($)**
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 20 =	_____ x	_____ =	_____

Multiple Dependent Claims

Fee ($)	**Fee Paid ($)**

Indep. Claims	**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
_____ - 3 =	_____ x	_____ =	_____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	**Extra Sheets**	**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
62 - 100 =	_____ /50	_____ (round up to a whole number) x	_____ =	_____

4. OTHER FEE(S) **Fees Paid ($)**

Non-English Specification, $130 fee (no small entity discount)

Other (e.g., late filing surcharge): _____

SUBMITTED BY

Signature	Anita Varma	Registration No. (Attorney/Agent)	43,221	Telephone	(617) 951-7796
Name (Print/Type)	Anita Varma			Date	May 11, 2005

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Dated: 5-11-05 Signature: Maura A. Gallagher (Maura A. Gallagher)

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Page 8133

17581 U.S.PTO 051105

PTO/SB/05 (04-05)
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UTILITY PATENT APPLICATION TRANSMITTAL

(ONLY FOR NEW NONPROVISIONAL APPLICATIONS UNDER 37 CFR 1.53(B))

Attorney Docket No.	ALEX-P01-001
First Inventor	Yi Wang
Title	NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES
Express Mail Label No.	EV543607732US

113243 U.S.PTO 11/127438 051105

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

ADDRESS TO: Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

1. [X] **Fee Transmittal Form** (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. [ ] **Applicant claims small entity status.** See 37 CFR 1.27.
3. [X] **Specification** [*Total Pages* 46]
   Both the claims and abstract must start on a new page
   (For information on the preferred arrangement, see MPEP 608.01(a))
4. [X] **Drawing(s)** *(35 U.S.C. 113)* [*Total Sheets* 16]
5. **Oath or Declaration** [*Total Sheets*]
   a. [ ] Newly executed (original or copy)
   b. [ ] A copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 18 completed)*
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. [X] **Application Data Sheet.** See 37 CFR 1.76
7. [ ] **CD-ROM or CD-R** in duplicate, large table or Computer Program *(Appendix)*
   [ ] Landscape Table on CD
8. **Nucleotide and/or Amino Acid Sequence Submission** *(if applicable, items a. – c. are required)*
   a. [ ] Computer Readable Form (CRF)
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ACCOMPANYING APPLICATION PARTS

9. [ ] **Assignment Papers** (cover sheet & document(s))
   Name of Assignee:
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[ ] Continuation [ ] Divisional [X] Continuation-in-part (CIP) of prior application No.: 10/655861

Prior application information: Examiner Not Yet Assigned *Art Unit:* N/A

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Address	One International Place				
City	Boston	State	MA	Zip Code	02110-2624
Country	US	Telephone	(617) 951-7000	Email	

Signature	Anita Varma	Date	May 11, 2005
Name (Print/Type)	Anita Varma	Registration No. (Attorney/Agent)	43,221

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Samsung Bioepis Exhibit No. 1002
Page 8134

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FEE TRANSMITTAL
For FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 1,000.00

Complete if Known	
Application Number	Not Yet Assigned
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Examiner Name	Not Yet Assigned
Art Unit	N/A
Attorney Docket No.	ALEX-P01-001

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☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

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☒ Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 ☒ Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	1,000.00
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Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
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Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____ - 20 =	_____	x _____	= _____

Multiple Dependent Claims: Fee ($) _____ Fee Paid ($) _____

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
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Non-English Specification, $130 fee (no small entity discount)

Other (e.g., late filing surcharge): _____

SUBMITTED BY

Signature	Anita Varma	Registration No. (Attorney/Agent)	43,221	Telephone	(617) 951-7796
Name (Print/Type)	Anita Varma			Date	May 11, 2005

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Dated: 5-11-05 Signature: Maura A. Gallagher (Maura A. Gallagher)

Samsung Bioepis Exhibit No. 1002
Page 8135

Nebulization of Monoclonal Antibodies for Treating Pulmonary Diseases

Related Applications

This application is a continuation-in-part application of U.S. Patent Application Serial No. 10/655,861, filed September 5, 2003, which claims priority to and benefit of the filing date of U.S. Provisional Application Serial No. 60/469,189, filed May 9, 2003 and U.S. Provisional Application Serial No. 60/408,571, filed September 6, 2002, all of which are incorporated by reference in their entirety.

Background

Asthma, bronchitis and emphysema are known collectively as Chronic Obstructive Pulmonary Diseases. These diseases are characterized as generalized airways obstruction, especially of small airways, associated with varying degrees of symptoms of chronic bronchitis, asthma, and emphysema. These diseases may often coexist in an individual, and it may be difficult to determine the primary cause of an airway obstruction. Airway obstruction is defined as an increased resistance to airflow during forced expiration. Obstruction of large airways may also occur in these diseases, particularly in asthma. Currently available therapy for asthma remains problematic. Similarly, improved therapy is also desirable for treating or preventing other pulmonary diseases, for example lung cancers.

Effective delivery to a patient is a critical aspect of any successful drug therapy. Various routes of delivery exist, and each has its own advantages and disadvantages. Oral drug delivery of pills, capsules, elixirs, and the like is perhaps the most convenient method, but many drugs are degraded in the digestive tract before they can be absorbed. Subcutaneous injection is frequently an effective route for systemic drug delivery, including the delivery of proteins, but enjoys a low patient acceptance. Since injection of drugs one or more times a day can frequently be a source of poor patient compliance, a variety of alternative routes of administration have also been developed, including transdermal, intranasal, intrarectal, intravaginal, and pulmonary delivery. Thus, it is desirable to improve drug delivery methods and compositions, particularly for antibody-based therapeutics in treating or preventing pulmonary diseases.

Summary

Accordingly, this application provides compositions and methods suitable for delivering antibody-based therapeutics. These antibody-based therapeutics can be particularly useful in preventing or treating pulmonary diseases or conditions such as for example asthma. Examples of antibodies useful in this application include anti-C5 antibody or antibodies that inhibit activation of the complement cascade, for example, the antibodies as described in U.S. Patent No. 6,355,245. In addition to antibodies, other therapeutics are also contemplated to be employed with the compositions and methods of the present application, for example, the chimeric complement inhibitor proteins as described in U.S. Patent No. 5,627,264. Certain preferred embodiments employ pexelizumab or eculizumab as the antibody therapeutic. In certain embodiments, the methods suitable for delivering antibody-based therapeutics for treating or preventing pulmonary diseases are not designed for systemic delivery of the therapeutics, and therefore, systemic effect of the therapeutics may not be observed using these methods.

A first aspect of the application provides a method for preventing or treating a pulmonary disease or condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade or blocks the function of one or more subsequently activated components of the complement system. In certain embodiments, an additional active agent is also administered to the same subject. Administration of the antibody and the additional active agent may occur simultaneously or sequentially in either order. In certain embodiments, the antibody and the additional active agent can be administered to the subject via the same delivery method or route, for example, by inhalation. In alternative embodiments, the antibody and the additional active agent can be administered to the subject via different delivery methods or routes, for example, the antibody delivered by inhalation and the additional active agent by injection or by oral intake.

A second aspect of the application provides an aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. The antibody is formulated in a composition suitable for aerosolization. The antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for aerosolization. Alternatively, the antibody and an additional active agent may be formulated

Samsung Bioepis Exhibit No. 1002
Page 8137

separately, such that they will be combined after aerosolization occurs or after being administered to a subject.

A third aspect of the application provides a nebulization composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. The antibody is formulated in a composition suitable for nebulization. Similarly, the antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for nebulization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after nebulization occurs or after being administered to a subject.

A further aspect of the application provides a biopharmaceutical package comprising an antibody that inhibits activation of the complement cascade and a nebulizer, wherein the package is suitable for preventing or treating a pulmonary disease or condition in a subject. The biopharmaceutical package may further comprise an active agent in addition to the antibody. The biopharmaceutical package may also comprise instructions for use.

Pulmonary diseases or conditions contemplated by the application include, but are not limited to, asthma, bronchial constriction, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

The timing of administering a therapeutic to a subject can vary, for example, depending on the identity of the subject or the pulmonary disease or condition to be treated or prevented, or both. For example, the administration may occur before the manifestation of the pulmonary condition (e.g., pre-asthmatic attack), during the manifestation of the pulmonary condition (e.g., during the asthmatic attack), or after the manifestation of the pulmonary condition (e.g., post-asthmatic attack).

An antibody of the present application can be specific to C5 such that it prevents the cleavage of C5 into C5a and C5b. The antibody can be specific to the C5 convertase. Alternatively, the antibody may be specific to a component of the complement system, for example, C5a, C5b, or C5b-9, and the antibody specific to the component preferably inhibits the

Samsung Bioepis Exhibit No. 1002
Page 8138

component's function, for example, by blocking the component's binding to its respective receptor, or by blocking its function in activating the subsequent signaling or events in the complement cascade. Certain embodiments employ eculizumab or pexelizumab, or both. An antibody or antibody therapeutic of the present application can be a full length immunoglobulin, a monoclonal antibody, a chimeric antibody (e.g., a humanized antibody), a single chain antibody, a domain antibody, an Fab fragment, or an antibody having an Fab fragment and a mutated Fc portion. In certain embodiments, the mutated Fc portion does not activate complement, or the mutation(s) in the Fc portion decreases the Fc portion's ability to activate complement. An antibody of the present application may be produced or processed in bulk and packaged in an ampule made of a suitable material (e.g., glass or plastic) at different doses.

An additional active agent (or an active agent in addition to the antibody therapeutic) of the present application can be another antibody therapeutic (e.g., an anti-IgE antibody such as Xolair® or omalizumab, an anti-IL-4 antibody or an anti-IL-5 antibody), an anti-IgE inhibitor (e.g., Singulair® or montelukast sodium), a sympathomimetic (e.g., albuterol), an antibiotic (e.g., tobramycin), a deoxyribonuclease (e.g., pulmozyme), an anticholinergic drug (e.g., ipratropium bromide), a corticosteroid (e.g., dexamethasone), a β-adrenoreceptor agonist, a leukotriene inhibitor (e.g., zileuton), a 5 Lipoxygenase inhibitor, a PDE inhibitor, a CD23 antagonist, an IL-13 antagonist, a cytokine release inhibitor, a histamine H1 receptor antagonist, an anti-histamine, an anti-inflammatory agent (e.g. cromolyn sodium) or a histamine release inhibitor.

An example of formulation suitable for aerosolization or nebulization of an antibody is in physiologic osmolarity (e.g., between 280 and 320 mM) at a suitable pH (e.g., pH 6 to 8). A formulation of the present application may further comprise an excipient, for example polysorbate 80 which can be used at 0.0015 to 0.02%.

A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC Jet Plus or Hudson T Up-draft II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., Micro air by Omron) and a shockwave nebulizer (EvitLabs Sonik LDI20).

Brief Description of the Drawings

The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

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Page 8139

Fig. 1 illustrates blocking the generation of C5a and C5b-9 by anti-C5 mAb, BB5.1. Pharmacodynamic profile (A) of inhibition of C5b-9 mediated hemolysis from animals given a single i.v. or i.p. injection of anti-C5 mAb (40 mg/kg) or control mAb (40 mg/kg). • represents data from 4 control animals that were given control mAb by either i.v. (n=2) or i.p. (n=2). ▲ represents i.p. anti-C5 mAb-treated animals (n=3). Δ represents i.v. anti-C5 mAb-treated animals (n=3). C5a-mediated neutrophil migration (B) was analyzed in an in vitro assay. Zymosan activated sera (20%) was used as the source of C5a. Anti-C5 mAb (100 μg/ml) was added to serum sample prior to zymosan activation as indicated (n=8). * indicates $p< 0.05$ when compared to zymosan activated serum samples. BALB/c mice were used in all figures except Figs. 4 C and D.

Fig. 2 shows airway response to OVA provocation and definition of three critical checkpoints. A: Representative DCP results indicating the longitudinal changes of sRaw. After aerosol challenge with 5% OVA for 10 min, the appearance of EAR was observed at 15 min, followed by the LAR at 5 h. B: Three critical points during the course of disease. Checkpoint 1: Anti-C5 mAb was given by i.p. injection on days 25, 29 and 31. Analysis of lower airway functions and quantification of airway inflammation were evaluated 5 h after aerosol challenge of 5% OVA on day 32. Checkpoint 2: Anti-C5 mAb was given by i.p. injection on day 33, after recovery from the first airway response on day 32. AHR was assessed by evaluating the changes of RL and Cdyn from baseline in response to aerosol Mch challenge on day 35. Checkpoint 3: C5 inhibition was given by either i.v. or aerosol administration of BB5.1 during EAR on day 35. Lower airway function was determined during the peak of LAR, 5 h after 5% OVA provocation.

Fig. 3 shows contribution of C5 at checkpoint 1, the initiation of airway inflammation. Mean serum OVA-specific antibody titers of 5-6 mice on day 32 were measured by ELISA (A). Cross hatched bars: OVA-specific IgG. Open bars: OVA-specific IgE. Also on day 32, animals were trachea cannulated for the measurement of RL (B) and Cdyn (C) 5 h after 5% aerosol OVA provocation, followed by histological analyses of lung (D). The mean histology score for control mAb-treated mice (F) is 2±0.28 compared to 0.64±0.21 for corticosteroid-treated animals (G) and 1.25±0.25 for anti-C5 mAb-treated animals (H) compared to total lack of inflammation, score =0, seen in sham-mice (E). * indicates $p<0.05$ when comparing treated animals (i.e. corticosteroid or anti-C5 mAb treatment) with control mAb treated animals, while ■ indicates $p<0.05$ when comparing corticosteroid treated mice with animals treated with antibody targeting

C5 or C5aR in this and all following figures unless otherwise indicated. For clarity, all control mAb treated animals are labeled as control in all following figures. Arrow indicates eosinophil infiltration. Giemsa stain, magnification = 100X.

Fig. 4 shows contribution of C5 at checkpoint 2, the development of AHR. C5s BALB/cByJ mice (A&B) and C5d B10D2oSn mice (C&D) were immunized with OVA in identical manner (Fig. 2 B). Animals were randomized and given (i.p.) the indicated treatment on day 33. On day 35, aerosol Mch challenges were administered through a trachea cannula during measurement of RL (A&C) and Cdyn (B&D). Changes in RL and Cdyn are expressed as a percentage of baseline after each aerosol challenge. Subgroups of C5d mice with or without OVA immunization were reconstituted (i.v.) with 200μg of rmC5a 3 hours prior to Mch challenges. For C5s BALB/c mice, * indicates $p<0.05$ when comparing either steroid or anti-C5 mAb treated animals with control mAb treated animals. For C5d B10D2oSn mice, * indicates $p<0.05$ when comparing the mAb treated animals (n=6) given either control mAb (n=3) or anti-C5 mAb (n=3) with OVA immunized C5d B10D2oSn mice reconstituted with rmC5a. There are no statistical differences between C5d animals treated with control mAb and anti-C5 mAb.

Fig. 5 shows contribution of C5 at checkpoint 3, during an on-going airway response. Animals that experienced previous airway response to allergen provocation on day 32 were given a second aerosol allergen challenge on day 35. During the peak of EAR, animals were randomized and given (i.v.) the indicated treatment. Animals were trachea cannulated for the measurement of RL (A) and Cdyn (B) 5 h after the OVA provocation. (n= 7-8 mice per group.)

Fig. 6 shows BALF WBC counts and differential analysis. BALF was obtained 5 h after aerosol allergen provocation at checkpoint 1 (A) or at checkpoint 3 (B&C). Total BALF WBC counts (A&B) and their differential analyses (C) were performed by a pathologist in a double blind fashion. For 6 (C), open bars: alveolar macrophages. Cross hatched bars: Eosinophils. Solid bars: Neutrophils. Hatched bars: Lymphocytes. (n= 6-8 mice per group.)

Fig. 7 shows BALF levels of inflammatory mediators. BALF was obtained 5 h after aerosol allergen provocation at checkpoint 1 (cross hatched bars) or checkpoint 3 (solid bars). BALF levels of IL-5 (A), IL-13 (B), histamine (C), eotaxin (D), RANTES (E), activated TGF-β (F), TNF-α (G) and proMMP9 (H) were measured by ELISA. (n= 6-8 samples per group.)

Samsung Bioepis Exhibit No. 1002
Page 8141

Fig. 8 shows contribution of intrapulmonary C5 activation at checkpoint 3. Results represent data pooled from 3 separate experiments with identical experimental procedures. Animals were aerosol challenged with 5% OVA and randomized. During EAR, animals were then given aerosol administration of one of the indicated treatments through a jet nebulizer. Sham-immunized mice were treated with aerosol PBS. All animals were trachea cannulated for the measurement of RL (A) and Cdyn (B) 5 h after the OVA provocation. BALF WBC counts (C) and proMMP9 (D) were analyzed in the majority of animals. The data from control mAb (n=15) and control sIgG treated mice (n=4) were pooled together. Except anti-C5 Fab (n=3), n=6-19 animals in all other cohorts.

Fig. 9 shows the result of combined therapy on the development of AHR in subjects with established airway inflammation. The results were obtained based on checkpoint 2 study as described herein. Treatment was delivered on day 32 by nebulization instead of i.p. Mch challenge occurred on day 35. For anti-C5, BB5.1 at 3 mg/ml was subjected to nebulization for 10 minutes. The steroid at 2 mg/ml was nebulized for 10 minutes. For the combination, the final concentration was 3 mg/ml for BB5.1 and 2 mg/ml for the steroid, which was subjected to nebulization for 10 minutes.

Fig. 10 shows exemplary formulations suitable for nebulization of an antibody.

Fig. 11 shows the result of Mch challenge two days after nebulization treatment.

Fig. 12 shows the particle size distribution.

Fig. 13 shows nebulization and delivery efficiencies.

Fig. 14 shows SDS-PAGE analysis of aerosolized eculizumab.

Fig. 15 shows SEC HPLC analysis of aerosolized eculizumab using two different nebulizers.

Fig. 16 shows that repeated long term aerosol administrations of anti-C5 monoclonal antibody do not induce airway inflammation. The mice here were subject to 10-week treatments: sham mice were treated with PBS for 10 min twice per week for 10 weeks; control mice were treated with HFN7.1 (an isotype-matched mouse antibody) for 10 min by nebulization, also twice a week for 10 weeks; the anti-C5 antibody (BB5.1) treated mice were treated with BB5.1 for 10 min by nebulization, also twice a week for 10 weeks.

Samsung Bioepis Exhibit No. 1002
Page 8142

Detailed Description of the Application

Overview

The present application abbreviates certain terms as follows:

AHR: airway hyper-responsiveness; BALF: bronchoalveolar lavage fluid; Cdyn - dynamic lung compliance; C5aR: C5a receptor; C5d: C5 deficient; C5s: C5 sufficient; DCP: double chamber plethysmograph; EAR: Early phase airway response; IC: immune complexes; LAR: Late phase airway response; Mch: methacholine; rmC5a: recombinant mouse C5a; RL: lung resistance; sRaw: Specific airway resistance; sIgG: serum IgG.

The Complement System

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the

Samsung Bioepis Exhibit No. 1002
Page 8143

circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2d ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner, et al., Complement Inflamm. 8:328-340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75 μg/ml (0.4 μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence.

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment and the alpha chain as a carboxyl terminal fragment, with four amino acids deleted between the two. C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain. Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733 of the 1659-amino acid pro-C5 precursor sequence with an 18-amino acid leader sequence (SEQ ID NO:2 as described in U.S. Patent No. 6,355,245). A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

Samsung Bioepis Exhibit No. 1002
Page 8144

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yamamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

C5a is another anaphylatoxin. C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex--TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

Pulmonary Diseases or Conditions, and the Complement System

Certain pulmonary diseases or conditions such as asthma can be characterized by a combination of chronic airway inflammation, airway obstruction and airway hyper-responsiveness (AHR) to various stimuli. It is thought to be mediated primarily by adaptive immune responses including allergen-specific $CD4^+$ T cells, Th2 cytokines, and allergen specific IgE leading to pulmonary inflammation and AHR. Complement and its activated components, which form a central core of innate immune defense against bacterial, viral and fungal invasions (Nagy et al., J. Allergy. Clin. Immunol. (2003) 112:729-734; Kasamatsu et al., Arerugi (1993) 42:1616-1622; Bjornson et al., Am. Rev. Respir. Dis. (1991) 143:1062-1066), can be activated through the classical pathway, alternative pathway and the lectin pathway (Lachmann, Res. Immunol. (1996) 147:69-70). All three activation pathways converge at complement component

Samsung Bioepis Exhibit No. 1002
Page 8145

C5 prior to the generation of C5a and C5b-9, both of which induce potent biological responses including tissue injury, inflammation, anaphylatoxic responses, and cell lysis at very low concentrations (Takafuji et al., Int. Arch. Allergy Immunol. (1994) 104 Suppl. 1:27-29). In addition, C5 can be activated after allergen exposure (Nagata et al., J. Allergy Clin. Immunol. (1987) 80:24-32). Recent data from animal models of allergic asthma suggest that activated complement components, such as C5a, provide a critical link between innate and adaptive immunity (Karp et al., Nat. Immunol. (2000) 1:221-226). However, controversies remain in the art regarding the involvement of C5 and its activated components in the pathogenesis of asthma (Karp et al., *supra*; Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708; Abe et al., J. Immunol. (2001) 167:4651-4660; Lukacs et al., Am. J. Physiol. Lung Cell. Mol. Physiol. (2001) 280(3):L512-L518).

Several experimental models for bronchial asthma have indicated that C5 and its activated components are involved in the development of airway inflammation and bronchoconstriction (or bronchial constriction). Inhibition studies with various complement inhibitors markedly reduced AHR or airway inflammation in rodents (Abe et al., *supra*; Lukacs et al., *supra*). The potential involvement of C5 activation was also extended to clinical observations that the severity of clinical symptoms was correlated with the extent of C5 activation (Gonczi et al., Allergy (1997) 52:1110-1114). On the other hand, studies have shown that C5 deficiency (C5d) leads to increased susceptibility to allergen-induced AHR in mice, and this finding is supported by evidence of decreased production of IL-12, a key Th1 cytokine reported to modulate the pathogenesis of asthma (Karp et al., *supra*). The key question at the center of the debate is whether C5 and its activated components are pro-inflammatory or anti-inflammatory during the sensitization phase and the effector phase of the pathogenesis as suggested respectively from studies of C5d animals (Karp et al., *supra*) and experiments with intervention during the course of disease (Abe et al., *supra*; Lukacs et al., *supra*). In addition, another key issue is whether intrapulmonary-activated complement components play a significant role in the pathogenesis and overcome the potential anti-inflammatory effect of activated C5 components on the adaptive immune system (Karp et al., *supra*).

The present application relates to the study of the contribution of C5 and its activated components at three critical points during the course of disease, which outlines the key mechanisms of this essential component of the innate immune system in the pathogenesis of

Samsung Bioepis Exhibit No. 1002
Page 8146

asthma. Comprehensive analyses of lower airway function and quantification of multiple parameters of airway inflammation have been conducted. As described herein, the study shows that C5 contributes to the initiation of airway inflammation, demonstrates the critical contribution of C5 in the development of AHR in animals with established airway inflammation and the significant role of activated C5 components in sustaining an on-going airway response to allergen challenge.

The pathophysiological hallmark necessary for the development of asthma-like symptoms is airway inflammation. Individuals with a genetic predisposition may have modulated adaptive immune responses to environmental exposures, including activation of allergen-specific $CD4^+$ T cells, polarization of Th2 cytokines, such as IL-4 and IL-5, which are critically involved in the production of allergen-specific IgE and the recruitment of eosinophils (Cieslewicz et al., J. Clin. Invest. (1999) 104:301-308). More recently, IL-13 was identified as a key player in the pathogenesis, and reported to be both necessary and sufficient to induce the airway response in animals (Wills-Karp et al., Science (1998) 282:2258-2261). As shown by the study described herein, the broad spectrum of anti-inflammatory activities of corticosteroid, including modulating the Th2 cytokine profile (Figs. 7 A and B) and blocking the recruitment of inflammatory cells into lower airways (Fig. 3 D), correlates with its potent antiasthmatic activity. Consistent with its anti-inflammatory activities reported earlier (Wang et al., Proc. Natl. Acad. Sci.U.S.A (1996) 93:8563-8568), C5 inhibition at checkpoint 1 resulted in significantly less airway inflammation without significant impact on the adaptive immune system's responses to allergen exposures. Further, C5d animals developed a similar degree of airway inflammation as that developed by C5s animals. One possible explanation is that airways are directly exposed to allergens and infections. These exposures may activate other complement components, such as C3a, which has very similar proinflammatory properties (Takafuji et al., Int. Arch. Allergy Immunol. (1994) 104 Suppl. 1:27-29; Nagata et al., J. Allergy Clin. Immunol. (1987) 80:24-32), and was known to contribute to the pathogenesis (Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708). The data provided in the present application indicate that activated C5 components are critically involved but are not necessary for the initiation of airway inflammation, which requires the activation of adaptive immune responses to aerosol exposures of allergen (Cieslewicz et al., *supra*). However, at checkpoints 2 and 3 when airway inflammation was already well established, the data described herein indicate that C5 inhibition has similar or

Samsung Bioepis Exhibit No. 1002
Page 8147

better efficacy than corticosteroid in ameliorating airway inflammation and improving lower airway functions (see, e.g., Figs. 4-8).

The second hallmark of asthma, the development of AHR in response to non-specific stimuli, was selected as another critical checkpoint. By pre-screening several strains of C5s and C5d mice, the genetic influences on the function of muscarinic acetylcholine receptor responsible for the intrinsic AHR (De Sanctis et al., Am. J. Respir. Crit. Care Med. (1997) 156:S82-S88; Levitt et al., FASEB J. (1988) 2:2605-2608.) are eliminated from the study described herein. Using animals that do not have intrinsic AHR, C5 inhibition is shown to be sufficient to prevent the development of AHR to aerosol Mch challenges in C5s BALB/c mice with established airway inflammation (Figs. 4 A and B) while reconstitution of C5d B10D2oSn mice with rmC5a restored AHR, also in the presence of established airway inflammation (Figs. 4 C and D). These data suggest that C5, most likely C5a, serves as a direct link between the innate immune responses and the key components of the airways responsible for AHR. C5aR was found on airway smooth muscle cells and airway epithelia (Drouin et al., J. Immunol. (2001) 166:2025-2032). Although the direct interaction of activated C5 components, in particular C5a, with receptors on airway smooth muscles and epithelium was demonstrated previously (Irvin et al., Am. Rev. Respir. Dis. (1986) 134:777-783; Larsen, Annu. Rev. Immunol. (1985) 3:59-85.), the data provided herein indicate that the maintenance of AHR depends upon complex interactions among airway epithelia, airway smooth muscle cells, inflammatory cells and their inflammatory mediators as suggested by minimal impact on the functions of lower airways during Mch challenges after systemic reconstitution (i.v.) of sham B10D2.oSn mice with rmC5a (Figs. 4 C and D).

The on-going airway responses after aerosol allergen provocation, serve as the third critical checkpoint in the study described herein. The development of EAR and LAR together with massive production and release of multiple inflammatory mediators provide a good opportunity to examine the complex interaction of activated C5 components with other components of innate and adaptive immune responses and their role in pathogenesis. The data described herein demonstrate that C5 inhibition by either anti-C5 monoclonal antibody (mAb) or its Fab fragment achieved similar in vivo efficacy in ameliorating intrapulmonary inflammatory activities as well as in improving functions of lower airways.

Samsung Bioepis Exhibit No. 1002
Page 8148

The data here also suggest that in the presence of airway inflammation, activated C5 components function as the key regulators of the downstream inflammatory cascade. This regulatory effect is demonstrated by its ability to influence the migration of inflammatory cells into airway lumen as well as the activation and the release of multiple harmful mediators corresponding to the significant changes of lower airway functions at checkpoint 3. C5a is probably the most potent activator of inflammatory cells (Takafuji et al., *supra*) and C5aR has been identified on circulating leukocytes, mast cells, macrophages, and endothelial cells (Chenoweth et al., Proc. Natl. Acad. Sci. U.S.A. (1978) 75:3943-3947). The disassociation of significant improvement of lower airway functions with significant presence of intrapulmonary inflammatory activities after anti-C5aR sIgG treatment is consistent with the hypothesis that the direct engagement of C5a with its receptors expressed on airway smooth muscle cells and epithelia is, in part, responsible for airway constriction (Irvin et al., *supra*). The data also indicate that the chemotactic activity and cell activation properties of C5b-9 (Czermak et al., Am. J. Pathol. (1999) 154:1513-1524) are responsible for the significant intrapulmonary inflammatory activities in the absence of C5aR engagement. In the presence of established airway inflammation and IC, C5b-9 can sufficiently regulate the downstream inflammatory cascade by triggering releases of multiple mediators such as proMMP9 independently of the engagement of C5a with its receptors expressed on inflammatory cells (Fig. 8 D). The presence of an elevated level of C5b-9 together with the presence of multiple inflammatory mediators contributed to migration of inflammatory cells into the bronchial lumen in animals treated with anti-C5aR sIgG (Fig. 8 C).

Both the adaptive and innate immune systems contributed in the complex process of the production and release of multiple inflammatory mediators, including leukotrienes, prostaglandins, histamine, alkaline proteins, cytokines, chemokines, and enzymes, which are responsible for bronchospasm, altered vascular permeability, adherence and migration of inflammatory cells, mucosal edema and excessive mucous secretion and ultimately lead to three unique but interdependent phenomena: airway inflammation, airway obstruction and AHR. Since there are many redundant biological mechanisms, under the right circumstances, such as in genetically deficient animals, these redundant mechanisms may compensate and contribute in a more significant fashion. The complex relationship and interactions of these components are

Samsung Bioepis Exhibit No. 1002
Page 8149

responsible for airway inflammation, obstruction and AHR, as well as other pulmonary diseases or conditions.

Lung parenchymal cells possess the ability to produce C5 and cleave C5 into activated fragments (Desai et al, J. Exp. Pathol. (1984) 1:201-216). The activation of C5 as part of innate immune responses may well be an innocuous by-product of a self-defensive response, due in part to direct enzymatic cleavage of C5 (Maruo et al, J. Allergy Clin. Immunol. (1997) 100:253-260) after exposure to allergens (Nagata et al., *supra*), house dust (Maruo et al., *supra*), smoke (Robbins et al., Am. J. Physiol. (1991) 260:L254-L259.) or airway infections (Nagy et al., J. Allergy. Clin. Immunol. (2003) 112:729-734; Kasamatsu et al., Arerugi (1993) 42:1616-1622; Bjornson et al., Am. Rev. Respir. Dis. (1991) 143:1062-1066). The formation of intrapulmonary IC after allergen exposure may also activate complement cascade (Larsen, *supra*). This hypothesis proposes that activated C5 components are primarily located at the epithelial side of the airway and, in non-asthmatic individuals, the intrapulmonary activation of complement is well regulated (Varsano et al., Thorax (2000) 55:364-369). Consistent with this hypothesis, our data demonstrated that intrapulmonary C5 inhibition achieved similar efficacy as systemic C5 inhibition at checkpoint 3 (Fig. 8) and at checkpoint 2. These data further suggest that intrapulmonary activated C5 components are major pro-inflammatory rather than anti-inflammatory forces.

The study also shows the effect of C5 inhibition on the inflammatory mediators produced or released by neutrophils or eosinophils (Fig. 7). These inflammatory mediators, in particular TGF-β, RANTES and proMMP9, may cause irreversible damage to airways by inducing bronchial smooth muscle hypertrophy and deposition of collagen under the basal membranes of respiratory epithelium, which leads to the remodel and repair of lower airways, the focal point for those individuals with a poor long term prognosis (Boulet et al., Am. J. Respir. Crit. Care Med. (2000) 162:1308-1313). Accordingly, C5 inhibition, which utilizes a unique and different anti-inflammatory mechanism, may complement the potent anti-inflammatory effect of corticosteroid and other active agents, and have an added benefit for long term prognosis.

This study clearly demonstrates that the complex functional changes of airways seen in individuals with asthma coexist with the complex inflammatory processes. On one hand, activated C5 components, such as C5a through its binding with airway C5aR, serve as the direct

Samsung Bioepis Exhibit No. 1002
Page 8150

link between the innate immune system and AHR. On the other hand, both C5a and C5b-9 regulate the downstream inflammatory cascade through their chemotactic and cell activation activities in the presence of IC and established airway inflammation. Blocking the generation of C5a and C5b-9 not only improves the functioning of lower airways but also ameliorates intrapulmonary inflammatory activities. In contrast, C5aR antagonist significantly improves functions of lower airways without a dramatic impact on intrapulmonary inflammatory activities.

Aerosol or Nebulization Compositions

In one respect, this application is directed to pulmonary drug delivery compositions and/or devices for delivering an antibody that inhibits the activation of the complement cascade. The pulmonary drug delivery compositions are useful for treating a pulmonary disease or condition. For example, aerosol compositions are provided for the delivery of an antibody or an antibody combined with an additional active agent to the respiratory tract. The respiratory tract includes the upper airways, including the oropharynx and larynx, followed by the lower airways, which include the trachea followed by bifurcations into the bronchi and bronchioli. The upper and lower airways are called the conductive airways. The terminal bronchioli then divide into respiratory bronchioli which then lead to the ultimate respiratory zone, the alveoli, or deep lung.

Pulmonary drug delivery may be achieved by inhalation, and administration by inhalation herein may be oral and/or nasal. Examples of pharmaceutical devices for pulmonary delivery include metered dose inhalers (MDIs), dry powder inhalers (DPIs), and nebulizers. Exemplary delivery systems by inhalation which can be adapted for delivery of the subject antibody and/or active agent are described in, for example, U.S. Patent Nos. 5,756,353; 5,858,784; and PCT applications WO98/31346; WO98/10796; WO00/27359; WO01/54664; WO02/060412. Other aerosol formulations that may be used for delivering the antibody and/or active agent are described in U.S. Patent Nos. 6,294,153; 6,344,194; 6,071,497, and PCT applications WO02/066078; WO02/053190; WO01/60420; WO00/66206.

Pressurized metered dose inhalers (pMDIs) are the most commonly used inhaler worldwide. The aerosol is created when a valve is opened (usually by pressing down on the propellant canister), allowing liquid propellant to spray out of a canister. Typically, a drug or therapeutic is contained in small particles (usually a few microns in diameter) suspended in the liquid propellant, but in some formulations the drug or therapeutic may be dissolved in the

Samsung Bioepis Exhibit No. 1002
Page 8151

propellant. The propellant evaporates rapidly as the aerosol leaves the device, resulting in small drug or therapeutic particles that are inhaled. Propellants typically used in such pMDIs include but are not limited to hydrofluoroalkanes (HFAs). A surfactant may also be used, for example, to formulate the drug or therapeutic, with pMDIs. Other solvents or excipients may also be employed with pMDIs, such as ethanol, ascorbic acid, sodium metabisulfate, glycerin, chlorobutanol, and cetylpyridium chloride. Such pMDIs may further include add-on devices such as, for example, spacers, holding chambers and other modifications.

Nebulizers produce a mist of drug-containing liquid droplets for inhalation. They are usually classified into two types: ultrasonic nebulizers and jet nebulizers. A new type of nebulizer is also available, which does not require ultrasound or air pressure to function. Single breath atomizers have also been developed (e.g., Respimat®), which is used to deliver a drug in a single inhalation and may be preferred because of less contamination. Jet nebulizers are more common and use a source of pressurized air to blast a stream of air through a drug-containing water reservoir, producing droplets in a complex process involving a viscosity-induced surface instability that leads to nonlinear phenomena in which surface tension and droplet breakup on baffles play a role. Ultrasonic nebulizers produce droplets by mechanical vibration of a plate or mesh. In either type of nebulizer, the drug is usually contained in solution in the liquid in the nebulizer and so the droplets being produced contain drug in solution. However, for some formulations (e.g., Pulmicort) the drug is contained in small particles suspended in the water, which are then contained as particles suspended inside the droplets being produced. Certain excipients are usually included in formulations suitable for nebulization, such as sodium chloride (e.g., to maintain isotonicity), mineral acids and bases (e.g., to maintain or adjust pH), nitrogen headspace sparging, benzalkonium chloride, calcium chloride, sodium citrate, disodium edtate, and polysorbate 80.

The third type of inhaler is the dry powder inhaler (DPI). In DPIs, the aerosol is usually a powder, contained within the device until it is inhaled. The therapeutic or drug is manufactured in powder form as small powder particles (usually a few millionths of a meter, or micrometers, in diameter). In many DPIs, the drug or therapeutic is mixed with much larger sugar particles (e.g., lactose monohydrate), that are typically 50-100 micrometers in diameter. The increased aerodynamic forces on the lactose/drug agglomerates improve entrainment of the drug particles upon inhalation, in addition to allowing easier filling of small individual powder doses. Upon

Samsung Bioepis Exhibit No. 1002
Page 8152

inhalation, the powder is broken up into its constituent particles with the aid of turbulence and/or mechanical devices such as screens or spinning surfaces on which particle agglomerates impact, releasing the small, individual drug powder particles into the air to be inhaled into the lung. The sugar particles are usually intended to be left behind in the device and/or in the mouth-throat.

One aspect of the application provides an aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. An aerosol antibody composition can be a composition comprising aerosolized antibody or a composition comprising an antibody in a formulation suitable for aerosolization. The antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for aerosolization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after aerosolization occurs or after being administered to a subject.

Another aspect of the application provides a nebulization composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary disease or condition in a subject. A nebulization antibody composition can be a composition comprising a nebulized antibody or a composition comprising an antibody in a formulation suitable for nebulization. Similarly, the antibody may be formulated in combination with an additional active agent, and the combination formulation is suitable for nebulization. Alternatively, the antibody and an additional active agent may be formulated separately, such that they will be combined after nebulization occurs or after being administered to a subject.

A further aspect of the application provides a biopharmaceutical package comprising an antibody that inhibits activation of the complement cascade and a nebulizer, wherein the package is suitable for preventing or treating a pulmonary disease or condition in a subject. The biopharmaceutical package may further comprise an active agent in addition to the antibody. The biopharmaceutical package may also comprise instructions for use.

An antibody of the present application can be specific to C5 such that it prevents the cleavage of C5 into C5a and C5b. The antibody can be specific to the C5 convertase. Alternatively, the antibody may be specific to a component of the complement system, for

example, C5a, C5b, or C5b-9, and the antibody specific to the component preferably inhibits the component's function, for example, by blocking the component's binding to its respective receptor, or by blocking its function in activating subsequent signaling or events in the complement cascade. Certain embodiments employ eculizumab or pexelizumab, or both. An antibody or antibody therapeutic of the present application can be a full length immunoglobulin, a chimeric antibody (e.g., a humanized antibody), a single chain antibody, a domain antibody (e.g., a domain antibody as developed by Domantis, defined as the smallest functional binding units of antibodies, corresponding to the variable regions of either the heavy (VH) or light (VL) chains of human antibodies), an Fab fragment, or an antibody having an Fab fragment and a mutated Fc portion. In certain embodiments, the mutated Fc portion does not activate complement, or the mutation(s) in the Fc portion decreases the Fc portion's ability to activate complement. An antibody of the present application may be produced or processed in bulk and packaged in an ampule made of a suitable material (e.g., glass or plastic) at different doses. The antibody may be stable in a formulation at a concentration ranging from 1 mg/ml to 200 mg/ml.

An additional active agent (or an active agent in addition to the antibody therapeutic) of the present application can be another antibody therapeutic (e.g., an anti-complement or anti-C5 antibody, an anti-IgE antibody such as Xolair® or omalizumab, an anti-IL-4 antibody or an anti-IL-5 antibody), an anti-IgE inhibitor (e.g., Singulair® or montelukast sodium), a sympathomimetic (e.g., albuterol), an antibiotic (e.g., tobramycin), a deoxyribonuclease (e.g., pulmozyme), an anticholinergic drug (e.g., ipratropium bromide), a corticosteroid (e.g., dexamethasone), a β-adrenoreceptor agonist, a leukotriene inhibitor (e.g., zileuton), a 5 Lipoxygenase inhibitor, a PDE inhibitor, a CD23 antagonist, an IL-13 antagonist, a cytokine release inhibitor, a histamine H1 receptor antagonist, an anti-histamine, an anti-inflammatory agent (e.g. cromolyn sodium) or a histamine release inhibitor. As used herein, an active agent may also be referred to as a therapeutic or a drug.

An example of formulation suitable for aerosolization or nebulization of an antibody is in physiologic osmolarity (e.g., between 280 and 320 mM) at a suitable pH (e.g., pH 6 to 8). A formulation of the present application may further comprise an excipient, for example polysorbate 80 which can be used at 0.0015 to 0.02%.

Samsung Bioepis Exhibit No. 1002
Page 8154

U.S. Patent No. 5,474,759 discloses aerosol formulations that are substantially free of chlorofluorocarbons, and having particular utility in medicinal applications. The formulations contain a propellant (such as 1,1,1,2,3,3,3,-heptafluoropropane), a medium-chain fatty acid propylene glycol diester, a medium-chain triglyceride, optionally a surfactant, and optionally auxiliary agents such as antioxidants, preservatives, buffers, sweeteners and taste masking agents.

Other pharmaceutically acceptable carriers may also be used in a formulation of the present application. The phrase "pharmaceutically acceptable" is employed herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio. The phrase "pharmaceutically acceptable carrier" as used herein means a pharmaceutically acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject antagonists from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Some examples of materials which can serve as pharmaceutically acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations.

Methods of preparing these formulations or compositions include the step of bringing into association a compound of the present application with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and

Samsung Bioepis Exhibit No. 1002
Page 8155

intimately bringing into association a compound of the present application with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC Jet Plus or Hudson T Up-draft II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., Micro air by Omron) and a shockwave nebulizer (EvitLabs Sonik LDI20).

"Aerosol composition" means an antibody and/or an active agent described herein in a form or formulation that is suitable for pulmonary delivery. The aerosol composition may be in the dry powder form, it may be a solution, suspension or slurry to be nebulized, or it may be in admixture with a suitable low boiling point, highly volatile propellant. It is to be understood that more than one antibody and optionally other active agents or ingredients may be incorporated into the aerosolized formulation or aerosol composition and that the use of the term "antibody" or "active agent" in no way excludes the use of two or more such antibodies or other agents or ingredients.

In certain preferred embodiments, an antibody or active agent retains more than 50% of its activity after nebulization, preferably more than 70%. In certain preferred embodiments, an antibody or active agent retains more than 50% of its purity after nebulization, preferably more than 70%.

Active agent formulations suitable for use in the present application include dry powders, solutions, suspensions or slurries for nebulization and particles suspended or dissolved within a propellant. Dry powders suitable for use in the present application include amorphous active agents, crystalline active agents and mixtures of both amorphous and crystalline active agents. The dry powder active agents have a particle size selected to permit penetration into the alveoli of the lungs, that is, preferably 10 μm mass median diameter (MMD), preferably less than 7.5 μm, and most preferably less than 5 μm, and usually being in the range of 0.1 μm to 5 μm in diameter. The delivered dose efficiency (DDE) of these powders is >30%, usually >40%, preferably >50 and often >60% and the aerosol particle size distribution is about 1.0-5.0 μm mass median aerodynamic diameter (MMAD), usually 1.5-4.5 μm MMAD and preferably 1.5-4.0 μm MMAD. These dry powder active agents have a moisture content below about 10% by weight, usually below about 5% by weight, and preferably below about 3% by weight. Such

Samsung Bioepis Exhibit No. 1002
Page 8156

active agent powders are described in WO 95/24183 and WO 96/32149, which are incorporated by reference herein.

Dry powder active agent formulations are preferably prepared by spray drying under conditions which result in a substantially amorphous powder. Bulk active agent, usually in crystalline form, is dissolved in a physiologically acceptable aqueous buffer, typically a citrate buffer having a pH range from about 2 to 9. The active agent is dissolved at a concentration from 0.01% by weight to 1% by weight, usually from 0.1% to 0.2%. The solutions may then be spray dried in a conventional spray drier available from commercial suppliers such as Niro A/S (Denmark), Buchi (Switzerland) and the like, resulting in a substantially amorphous powder. These amorphous powders may also be prepared by lyophilization, vacuum drying, or evaporative drying of a suitable active agent solution under conditions to produce the amorphous structure. The amorphous active agent formulation so produced can be ground or milled to produce particles within the desired size range. Dry powder active agents may also be in a crystalline form. The crystalline dry powders may be prepared by grinding or jet milling the bulk crystalline active agent.

The active agent powders of the present application may optionally be combined with pharmaceutical carriers or excipients which are suitable for respiratory and pulmonary administration. Such carriers may serve simply as bulking agents when it is desired to reduce the active agent concentration in the powder which is being delivered to a patient, but may also serve to improve the dispersability of the powder within a powder dispersion device in order to provide more efficient and reproducible delivery of the active agent and to improve handling characteristics of the active agent such as flowability and consistency to facilitate manufacturing and powder filling. Such excipients include but are not limited to (a) carbohydrates, e.g., monosaccharides such as fructose, galactose, glucose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, cellobiose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; (b) amino acids, such as glycine, arginine, aspartic acid, glutamic acid, cysteine, lysine, and the like; (c) organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, magnesium gluconate. sodium gluconate, tromethamin hydrochloride, and the like; (d) peptides and proteins such as aspartame, human serum albumin, gelatin, and the like; and (e) alditols, such as mannitol, xylitol, and the like. A preferred group of

Samsung Bioepis Exhibit No. 1002
Page 8157

carriers includes lactose, trehalose, raffinose, maltodextrins, glycine, sodium citrate, human serum albumin and mannitol.

The dry powder active agent formulations may be delivered using Inhale Therapeutic Systems' dry powder inhaler as described in WO 96/09085 which is incorporated herein by reference, but adapted to control the flow rate at a desirable level or within a suitable range. The dry powders may also be delivered using a metered dose inhaler as described by Laube et al. in U.S. Patent No. 5,320,094, which is incorporated by reference herein.

Nebulized solutions may be prepared by aerosolizing commercially available active agent formulation solutions. These solutions may be delivered by a jet nebulizer such as the Raindrop, produced by Puritan Bennett, the use of which is described by Laube et al., *supra*. Other methods for delivery of solutions, suspensions of slurries are described by Rubsamen et al, U.S. Patent No. 5,672,581. A device that uses a vibrating, piezoelectric member is described in Ivri et al., U.S. Patent No. 5,586,550, which is incorporated by reference herein.

Propellant systems may include an active agent dissolved in a propellant or particles suspended in a propellant. Both of these types of formulations are described in Rubsamen et al., U.S. Patent No. 5,672,581, which is incorporated herein by reference.

In certain embodiments, an aerosol or nebulization antibody composition can be combined with one or more other aerosol or nebulization treatments, such as sympathomimetics (e.g., albuterol), antibiotics (e.g., tobramycin), deoxyribonucleases (e.g., pulmozyme), anticholinergic drugs (e.g., ipratropium bromide), or corticosteroids.

In certain embodiments, an aerosol or nebulization antibody composition can be combined with one or more other therapies (concurrently or sequentially) administered via nebulization, inhalation, intravenous or oral routes, such as sympathomimetics (e.g., albuterol), anticholinergic drugs (e.g., ipratropium bromide), inflammation inhibitors (e.g., cromolyn sodium), leukotriene inhibitors (e.g., zileuton), anti-IgE inhibitors (e.g., Singulair®) or corticosteroids.

As described herein, an antibody or therapeutic may be formulated as microparticles. Microparticles having a diameter of between 0.5 and 10 microns can penetrate the lungs, passing

through most of the natural barriers. A diameter of less than ten microns is generally required to bypass the throat; a diameter of 0.5 microns or greater is usually required to avoid being exhaled.

In certain embodiments, the subject antibody or therapeutic is formulated in a supramolecular complex, which may have a diameter of between 0.5 and 10 microns, which can be aggregated into particles having a diameter of between 0.5 and 10 microns.

In other embodiments, the subject antibodies or therapeutics are provided in liposomes or supramolecular complexes appropriately formulated for pulmonary delivery.

(i). Polymers for forming Microparticles

In addition to the supramolecular complexes described above, a number of other polymers can be used to form the microparticles. As used herein, the term "microparticles" includes microspheres (uniform spheres), microcapsules (having a core and an outer layer of polymer), and particles of irregular shape.

Polymers are preferably biodegradable within the time period over which release of the antibody or therapeutic is desired or relatively soon thereafter, generally in the range of one year, more typically a few months, even more typically a few days to a few weeks. Biodegradation can refer to either a breakup of the microparticle, that is, dissociation of the polymers forming the microparticles and/or of the polymers themselves. This can occur as a result of change in pH from the carrier in which the particles are administered to the pH at the site of release, as in the case of the diketopiperazines, hydrolysis, as in the case of poly(hydroxy acids), by diffusion of an ion such as calcium out of the microparticle, as in the case of microparticles formed by ionic bonding of a polymer such as alginate, and by enzymatic action, as in the case of many of the polysaccharides and proteins. In some cases linear release may be most useful, although in others a pulse release or "bulk release" may provided more effective results.

Representative synthetic materials are: diketopiperazines, poly(hydroxy acids) such as poly(lactic acid), poly(glycolic acid) and copolymers thereof, polyanhydrides, polyesters such as polyorthoesters, polyamides, polycarbonates, polyalkylenes such as polyethylene, polypropylene, poly(ethylene glycol), poly(ethylene oxide), poly(ethylene terephthalate), poly vinyl compounds such as polyvinyl alcohols, polyvinyl ethers, polyvinyl esters, polyvinyl halides, polyvinylpyrrolidone, polyvinylacetate, and poly vinyl chloride, polystyrene, polysiloxanes,

Samsung Bioepis Exhibit No. 1002
Page 8159

polymers of acrylic and methacrylic acids including poly(methyl methacrylate), poly(ethyl methacrylate), poly(butylmethacrylate), poly(isobutyl methacrylate), poly(hexylmethacrylate), poly(isodecyl methacrylate), poly(lauryl methacrylate), poly(phenyl methacrylate), poly(methyl acrylate), poly(isopropyl acrylate), poly(isobutyl acrylate), poly(octadecyl acrylate), polyurethanes and co-polymers thereof, celluloses including alkyl cellulose, hydroxyalkyl celluloses, cellulose ethers, cellulose esters, nitro celluloses, methyl cellulose, ethyl cellulose, hydroxypropyl cellulose, hydroxy-propyl methyl cellulose, hydroxybutyl methyl cellulose, cellulose acetate, cellulose propionate, cellulose acetate butyrate, cellulose acetate phthalate, carboxylethyl cellulose, cellullose triacetate, and cellulose sulphate sodium salt, poly(butic acid), poly(valeric acid), and poly(lactide-co-caprolactone).

Natural polymers include alginate and other polysaccharides including dextran and cellulose, collagen, albumin and other hydrophilic proteins, zein and other prolamines and hydrophobic proteins, copolymers and mixtures thereof. As used herein, chemical derivatives thereof refer to substitutions, additions of chemical groups, for example, alkyl, alkylene, hydroxylations, oxidations, and other modifications in the art.

Bioadhesive polymers include bioerodible hydrogels described by H. S. Sawhney, C. P. Pathak and J .A. Hubell in Macromolecules, 1993, 26, 581-587, polyhyaluronic acids, casein, gelatin, glutin, polyanhydrides, polyacrylic acid, alginate, chitosan, and polyacrylates.

To further illustrate, the matrices can be formed of the polymers by solvent evaporation, spray drying, solvent extraction and other methods known to those skilled in the art. Methods developed for making microspheres for drug delivery are described in the literature, for example, as described by Mathiowitz and Langer, J. Controlled Release 5,13-22 (1987); Mathiowitz, et al., Reactive Polymers 6, 275-283 (1987); and Mathiowitz, et al., J. Appl. Polymer Sci. 35, 755-774 (1988). The selection of the method depends on the polymer selection, the size, external morphology, and crystallinity that is desired, as described, for example, by Mathiowitz, et al., Scanning Microscopy 4,329-340 (1990); Mathiowitz, et al., J. Appl. Polymer Sci. 45, 125-134 (1992); and Benita, et al., J. Pharm. Sci. 73, 1721-1724 (1984).

In solvent evaporation, described for example, in Mathiowitz, et al., (1990), Benita, and U.S. Pat. No. 4,272,398 to Jaffe, the polymer is dissolved in a volatile organic solvent. The antibody and/or therapeutic, either in soluble form or dispersed as fine particles, is added to the

Samsung Bioepis Exhibit No. 1002
Page 8160

polymer solution, and the mixture is suspended in an aqueous phase that contains a surface active agent such as poly(vinyl alcohol). The resulting emulsion is stirred until most of the organic solvent evaporates, leaving solid microspheres.

In general, the polymer can be dissolved in methylene chloride. Several different polymer concentrations can be used, for example, between 0.05 and 0.20 g/ml. After loading the solution with drug, the solution is suspended in 200 ml of vigorously stirring distilled water containing 1% (w/v) poly(vinyl alcohol) (Sigma Chemical Co., St. Louis, Mo.). After four hours of stirring, the organic solvent will have evaporated from the polymer, and the resulting microspheres will be washed with water and dried overnight in a lyophilizer.

Microspheres with different sizes (1-1000 microns, though less than 10 microns for aerosol applications) and morphologies can be obtained by this method which is useful for relatively stable polymers such as polyesters and polystyrene. However, labile polymers such as polyanhydrides may degrade due to exposure to water. For these polymers, hot melt encapsulation and solvent removal may be preferred.

In hot melt encapsulation, the polymer is first melted and then mixed with the solid particles of antibodies or therapeutics, preferably sieved to appropriate size. The mixture is suspended in a non-miscible solvent such as silicon oil and, with continuous stirring, heated to 5 °C above the melting point of the polymer. Once the emulsion is stabilized, it is cooled until the polymer particles solidify. The resulting microspheres are washed by decantation with petroleum ether to give a free-flowing powder. Microspheres with diameters between one and 1000 microns can be obtained with this method. The external surface of spheres prepared with this technique are usually smooth and dense. This procedure is useful with water labile polymers, but is limited to use with polymers with molecular weights between 1000 and 50000.

In spray drying, the polymer is dissolved in an organic solvent such as methylene chloride (0.04 g/ml). A known amount of antibody and/or therapeutic is suspended (if insoluble) or co-dissolved (if soluble) in the polymer solution. The solution or the dispersion is then spray-dried. Microspheres ranging in diameter between one and ten microns can be obtained with a morphology which depends on the selection of polymer.]

Hydrogel microspheres made of gel-type polymers such as alginate or polyphosphazines or other dicarboxylic polymers can be prepared by dissolving the polymer in an aqueous

Samsung Bioepis Exhibit No. 1002
Page 8161

solution, suspending the material to be incorporated into the mixture, and extruding the polymer mixture through a microdroplet forming device, equipped with a nitrogen gas jet. The resulting microspheres fall into a slowly stirring, ionic hardening bath, as described, for example, by Salib, et al., Pharmazeutische Industrie 40-111A, 1230 (1978). The advantage of this system is the ability to further modify the surface of the microspheres by coating them with polycationic polymers such as polylysine, after fabrication, for example, as described by Lim, et al., J. Pharm. Sci. 70, 351-354 (1981). For example, in the case of alginate, a hydrogel can be formed by ionically crosslinking the alginate with calcium ions, then crosslinking the outer surface of the microparticle with a polycation such as polylysine, after fabrication. The microsphere particle size will be controlled using various size extruders, polymer flow rates and gas flow rates.

Chitosan microspheres can be prepared by dissolving the polymer in acidic solution and crosslinking with tripolyphosphate. For example, carboxymethylcellulose (CMC) microsphere are prepared by dissolving the polymer in an acid solution and precipitating the microspheres with lead ions. Alginate/polyethyleneimine (PEI) can be prepared to reduce the amount of carboxyl groups on the alginate microcapsules.

(ii). Pharmaceutical Compositions

The microparticles can be suspended in any appropriate pharmaceutical carrier, such as saline, for administration to a patient. In the most preferred embodiment, the microparticles will be stored in dry or lyophilized form until immediately before administration. They can then be suspended in sufficient solution, for example an aqueous solution for administration as an aerosol, or administered as a dry powder.

(iii). Targeted Administration

The microparticles can be delivered to specific cells or organ, especially phagocytic cells and organs. Where systemic delivery of the drug or therapeutic is desirable, endocytosis of the microparticles by macrophages in the lungs can be used to target the microparticles to the spleen, bone marrow, liver, lymph nodes, and other parts of the body.

The microparticles can also be targeted by attachment of ligands, such as those described above, which specifically or non-specifically bind to particular targets, for example, particular targets within the pulmonary system or associated with a pulmonary disease or condition (e.g., a target specific for cancerous cells in the lung). Examples of such ligands also include antibodies

Samsung Bioepis Exhibit No. 1002
Page 8162

and fragments including the variable regions, lectins, and hormones or other organic molecules having receptors on the surfaces of the target cells.

(iv). Storage of the Microparticles

In certain embodiments, the microparticles are stored lyophilized. The dosage is determined by the amount of encapsulated antibodies and/or therapeutics, the rate of release within the pulmonary system, and the pharmacokinetics of the antibodies and/or therapeutics.

(v). Delivery of Microparticles

The microparticles can be delivered using a variety of methods, ranging from administration directly into the nasal passages so that some of the particles reach the pulmonary system, to the use of a powder instillation device, to the use of a catheter or tube reaching into the pulmonary tract. As described above, dry powder inhalers are commercially available, although those using hydrocarbon propellants are no longer used and those relying on the intake of a breath by a patient can result in a variable dose. Examples of suitable propellants include hydrofluoroalkane propellants, such as 1,1,1,2-tetrafluoroethane (CF3CH2F) (HFA-134a) and 1,1,1,2,3,3,3-heptafluoro-n-propane (CF3CHFCF3) (HFA-227), perfluoroethane, monochloroifluoromethane, 1,1 difluoroethane, and combinations thereof.

Therapeutic Methods

A first aspect of the application provides a method for preventing or treating a pulmonary disease or condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade. In certain embodiments, an additional active agent is also administered to the same subject. Administration of the antibody and the additional active agent may occur simultaneously or sequentially in either order.

The term "preventing" is art-recognized, and when used in relation to a condition, such as a local recurrence (e.g., pain), a disease such as cancer, a syndrome complex such as heart failure or any other medical condition, is well understood in the art, and includes administration of a composition which reduces the frequency of, or delays the onset of, symptoms of a medical condition (e.g., asthma) in a subject relative to a subject which does not receive the composition. Thus, prevention of cancer includes, for example, reducing the number of detectable cancerous growths in a population of patients receiving a prophylactic treatment relative to an untreated

Samsung Bioepis Exhibit No. 1002
Page 8163

control population, and/or delaying the appearance of detectable cancerous growths in a treated population versus an untreated control population, e.g., by a statistically and/or clinically significant amount. Prevention of an infection includes, for example, reducing the number of diagnoses of the infection in a treated population versus an untreated control population, and/or delaying the onset of symptoms of the infection in a treated population versus an untreated control population. Prevention of pain includes, for example, reducing the magnitude of, or alternatively delaying, pain sensations experienced by subjects in a treated population versus an untreated control population.

The term "treating" includes prophylactic and/or therapeutic treatments. The term "prophylactic or therapeutic" treatment is art-recognized and includes administration to the host of one or more of the subject compositions. If it is administered prior to clinical manifestation of the unwanted condition (e.g., disease or other unwanted state of the host animal) then the treatment is prophylactic, (i.e., it protects the host against developing the unwanted condition), whereas if it is administered after manifestation of the unwanted condition, the treatment is therapeutic, (i.e., it is intended to diminish, ameliorate, or stabilize the existing unwanted condition or side effects thereof).

Pulmonary diseases or conditions contemplated by the application include, but are not limited to, asthma, bronchial constriction, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

The subject or patient receiving this treatment is any animal in need, including primates, in particular humans, and other mammals such as equines, cattle, swine and sheep, and poultry and pets in general.

The phrase "therapeutically effective amount" as used herein means that amount of a compound, material, or composition comprising a compound of the present invention which is effective for producing some desired therapeutic effect by inhibiting C5 or activation of the complement cascade in at least a sub-population of cells or an organ in an animal and thereby blocking the biological consequences of that function in the treated cells or an organ, at a reasonable benefit/risk ratio applicable to any medical treatment.

Samsung Bioepis Exhibit No. 1002
Page 8164

The timing of administering a therapeutic to a subject can vary, for example, depending on the identity of the subject or the pulmonary disease or condition to be treated or prevented, or both. For example, the administration may occur before the manifestation of the pulmonary condition (e.g., pre-asthmatic attack or pre-bronchial constriction), during the manifestation of the pulmonary condition (e.g., during the asthmatic attack or bronchial constriction), or after the manifestation of the pulmonary condition (e.g., post-asthmatic attack or post-bronchial constriction).

The phrase "inhibits the activation of complement cascade" refers to any inhibitory effect or action on any component of the complement system as described above conferred by an antibody or active agent of the present application. The inhibitory effect may be manifested by decrease in or lack of a response typical to activation of complement. For example, typical cellular and other biological responses to activation of complement include, but are not limited to, release of histamine, smooth muscle contraction, increased vascular permeability, leukocyte activation, chemotaxis (e.g., movement of granulocytes to the site of complement activation), and other inflammatory phenomena including cellular proliferation. In certain embodiments, the inhibitory effect is specific to the C5 component. In certain embodiments, the inhibitory effect is specific to C5 convertase, for example, blocking C5 convertase's function to convert C5 into C5a and C5b-9. In certain embodiments, the inhibitory effect is specific to C5a. In certain embodiments, the inhibitory effect is specific to C5b or C5b-9.

Examples

The following examples are offered by way of illustration and not by way of limitation.

Materials and Methods

Animals: Male C5s BALB/cByJ, C57BL/6J mice and C5d B10D2oSn/J, AKR/J and SWR/J mice were purchased from the Jackson Laboratory and housed in a pathogen free facility. In addition to serum C5b-9 mediated hemolysis, the C5 genotype was further confirmed by PCR performed on tail DNA using a pair of primers (5'-CAC GAT AAT GGG AGT CAT CTG GG-3' (SEQ ID NO:1) and 5'-AAG TTG GAG TGT GGT CTT TGG GCC-3' (SEQ ID NO:2) that amplify a 280-bp DNA fragment from both C5s and C5d DNA. This fragment encodes a HindIII site that is selectively destroyed by a mutation in the C5 gene, such that HindIII (New England BioLabs,MA) digestion selectively cleaves the C5s but not the C5d PCR products into

Samsung Bioepis Exhibit No. 1002
Page 8165

150 and 130-bp fragments. All animal protocols were reviewed by Institutional Committee and were in accordance with NIH guidelines.

Reagents: Anti-mouse C5 mAb (BB5.1) (Wang et al., Proc. Natl. Acad. Sci. U.S.A (1996) 93:8563-8568) and an isotype matched irrelevant control mAb (HFN7.1), which is specific to human fibronectin, were purified from ascites. BB5.1 Fab, which was purified with a kit (Pierce Biotechnology), had retained similar activity of BB5.1 and was more than 97% in purity. The anti-C5aR sIgG, which was shown to prevent mortality in experimental sepsis (Riedemann et al., J. Clin. Invest. (2002) 110:101-108), was able to prevent zymosan induced neutrophil migration. Both anti-C5aR sIgG and the control sIgG were purified from sera harvested from C5aR deficient mice (Gerard et al., Curr. Opin. Immunol. (2002) 14:705-708) that were repeatedly immunized with either PBS or peptide spanning the N-terminus of the mouse C5aR and one extra cysteine (Riedemann et al., *supra*) emulsified with CFA. Antibodies from either ascites or serum samples were purified by protein A affinity chromatography (Amersham). Corticosteroid (dexamethasone) was purchased from Sigma. Recombinant mouse C5a, which was free of endotoxin contamination and had potent chemotactic activity in neutrophil migration assay, was cloned and purified as previously reported (Riedemann et al., *supra*).

Induction of Airway Inflammation and Severe Airway Response

BALB/c and B10D2oSn Mice 10-12 wks of age were sensitized on day 1 and day 14 with i.p. injection of 20 μg OVA (Grade V; Sigma) emulsified in 2 mg aluminum hydroxide (Alum Inject; Pierce Biotechnology). Mice were immunized with 1% OVA for 10 min via airway on days 28, 29 and 30 with a jet nebulizer (Harvard Apparatus) connected to a single housing chamber (Buxco Electronics, Inc.). On day 32, all sensitized animals were aerosol challenged with 5% OVA for 10 min. In checkpoint 3 experiments, BALB/c mice were aerosol challenged with 5% OVA again on day 35. Sham C5s or C5d mice were immunized with Alum only and aerosol challenged with PBS instead of OVA.

Non-Invasive Measurement of sRaw

sRaw, which measures the resistance of both upper and lower airways, was measured by a DCP in conscious animals with spontaneous breathing (Buxco). The DCP was used to monitor

Samsung Bioepis Exhibit No. 1002
Page 8166

the longitudinal changes of sRaw prior to and after each 5% OVA aerosol challenge. In checkpoint 3 experiments, a DCP was used to ensure the appearance of EAR prior to randomizing animals into different treatment groups.

Invasive Measurement of Lower Airway Functions

Changes in RL and Cdyn were measured by using Buxco Biosystem software and a Buxco whole body plethysmograph. Mice were anesthetized (Avertin, 160 mg/kg) and tracheas cannulated. Spontaneous breathing was blocked by pancuronium bromide (0.3 mg/kg i.p.). Respirations were maintained by a Harvard Apparatus Inspira ventilator, which calculated a tidal volume and respiratory rate according to body weight. Measurements of RL and Cdyn were performed during the peak of LAR, 5 h after aerosol challenge with 5% OVA. Real-time changes of RL and Cdyn were recorded by Buxco software and reported as the mean value of five min of recording. AHR was evaluated based on changes of RL and Cdyn during aerosol Mch challenges. Changes in RL and Cdyn were expressed as a percentage of baseline after each aerosol challenge. A Buxco aerosol control and sonicating nebulizer unit was attached to the ventilator for aerosol delivery of Mch through tracheal cannulation. PBS or Mch (1.6 mg/ml) was delivered at the rate of 10 puffs or 20 puffs per 10 seconds, with each puff of aerosol delivery lasting 15 ms.

C5 Inhibition

(1) For C5 inhibition at checkpoint 1, anti-C5 mAb (BB5.1) or a control mAb (HFN7.1) was administered i.p. at 40 mg/kg on days 25, 29, and 31. Dexamethasone (2 mg/kg) was used as a positive control. (2) Animals that experienced airway response to 5% OVA provocation on day 32 were randomized for the following studies. For C5 inhibition at checkpoint 2, animals were given one i.p. injection of either dexamethasone (2 mg/kg), anti-C5 mAb (40 mg/ml) or control mAb (40 mg/kg) on day 33. On day 35, animals were anesthetized and tracheas cannulated for measurement of RL and Cdyn during aerosol Mch challenges. (3) At checkpoint 3, anti-C5 mAb (40 mg/kg), control mAb (40 mg/kg), or dexamethasone (2 mg/kg) was administered by i.v. injection 20 min after aerosol challenging animals with 5% OVA on day 35. For intrapulmonary C5 inhibition at this critical point, animals were given aerosol administration of either anti-C5 mAb (3 mg/ml), anti-C5 Fab (3 mg/ml), control mAb (3 mg/ml), anti-C5aR sIgG (3 mg/ml), control sIgG (2 mg/ml), or corticosteroid (2 mg/ml) for 10-30 min by using a jet

Samsung Bioepis Exhibit No. 1002
Page 8167

nebulizer connected to a single chamber housing the animal. Sham-immunized mice were given sham treatment with the same volume of PBS solution administered through the same route. The results from three intrapulmonary C5 inhibition experiments, which were conducted with identical protocols, were pooled together.

Analysis of Bronchoalveolar Lavage Fluid (BALF)

Approximately 5.5 h after allergen provocation, after the measurement of RL and Cdyn, BALF was harvested by instilling 1 ml PBS through the tracheal cannula, followed by gentle aspiration. The BALF was resuspended in 400 μl PBS. The total numbers of WBC in BALF were counted by using a hemocytometer or an automatic cell counter as in the aerosol experiments (Cell-Dyn 3700 Abbott). Cytospin slides were prepared, fixed and stained using Diff-Quik (VWR International, Inc.). The WBC differential was determined by a certified pathologist after counting a total of 300 WBC per slide on a 100x microscopic lens. The BALF levels of proMMP9, activated TGF-β, RANTES, eotaxin and IL-13 (R&D Systems, Inc.), IL-5 (Amersham Pharmacia Biotech, Inc.), and histamine (Immunotech, Inc.) were measured by ELISA according to the manufacturer's instructions.

Analysis of Serum OVA Specific Antibodies

Serum samples were harvested 5 h after 5% allergen provocation on day 32 for OVA specific IgG and IgE by ELISA.

Lung Histology

Lung was inflated with 10% buffered formalin (1 ml) through tracheal cannulation and fixed in 10% formalin at least 24 h. Lung samples were stained with Giemsa. Double blind histological analysis was performed to quantify airway inflammation according to the following criteria. 0: No detectable airway inflammation; 1: Less than 25% bronchials and surrounding vasculature were found with either perivascular or peribronchial inflammatory cell infiltration; 2: Approximately 25-50% of bronchials and surrounding vasculature were affected; 3: Approximately 50-75% bronchials and surrounding vasculature were affected; 4: >75% of bronchials and surrounding vasculature were affected.

C5b-9 Mediated Hemolytic Assay and in Vitro C5a Mediated Neutrophil Migration Assay

Samsung Bioepis Exhibit No. 1002
Page 8168

Serum harvested at the indicated time was used as the source of C5b-9, which mediated the hemolysis of chicken RBC as previously reported (Wang et al., *supra*). Mouse sera (Sigma) were incubated with either BB5.1 (100 μg/ml) or the same volume of PBS prior to activation with zymosan (1 mg/ml; Sigma). Diluted sera (20%) were used as the source of C5a for neutrophil chemotaxis experiments.

Statistical Analysis

The data were expressed as the means ± S.E.M. Student's one-tailed t-test assuming equal variance was used (MS Windows). A P value less than 0.05 was considered significant.

Blocking the generation of C5a and C5b-9 by anti-C5 mAb, BB5.1

Functional inhibition of complement component C5 by anti-C5 mAb was determined by the pharmacodynamic profile of blocking serum C5b-9 mediated hemolytic activity (Fig. 1 A). In addition, C5a mediated neutrophil migration was determined in an in vitro assay (Fig. 1 B). The pharmacodynamics of C5 inhibition by a single i.v. injection of anti-C5 mAb, BB5.1, is very different from the pharmacodynamics of a single i.p. injection during the first 5 h after administration. More than 80% of C5b-9 mediated hemolysis was inhibited within 1 h of i.v. administration of BB5.1, compared to less than 30% of inhibition by i.p. injection. Administration of anti-C5 mAb continued blocking more than 60% of hemolysis between 5 h and 48 h after i.v. injection while approximately 56% of hemolysis was blocked during this period of time after i.p. injection. Serum samples harvested from control mAb treated mice had more than 80% of normal hemolytic activity during the entire period of the study (Fig. 1 A). Hemolytic activity gradually returned to the normal range one wk after a single injection of anti-C5 mAb.

An in vitro neutrophil migration assay was employed to test the ability of anti-C5 mAb on blocking the generation of C5a (Fig. 1 B). Inhibition of serum samples with anti-C5 mAb prior to zymosan activation significantly blocked human neutrophil chemotaxis which was mediated by the presence of C5a after zymosan activation, as seen in zymosan activated control sera (Fig.1, B). Since anti-C5 mAb, BB5.1, blocked the generation of both C5a and C5b-9 and did not bind to C5a directly, this anti-C5 mAb is probably specific to blocking cleavage of C5 by C5 convertase.

Samsung Bioepis Exhibit No. 1002
Page 8169

Development of Severe Airway Response in OVA Sensitized Animals and Definition of Three Critical Points

OVA-sensitized animals immediately developed a severe airway response after aerosol challenge with 5% OVA (Fig. 2 A). A typical airway response to aerosol provocation with allergen consisted of an early-phase airway response (EAR), which is known to be mediated by the release of histamine by mast cells, and a late-phase airway response (LAR), which is mediated by the combination of effects of infiltration of inflammatory cells, edema and bronchial constriction (Larsen, Annu. Rev. Immunol. (1985) 3:59-85). The airway response after 5% OVA provocation was monitored by a non-invasive double chamber plethysmograph (DCP) in each mouse to measure the longitudinal changes of specific airway response (sRaw), which indicates the appearance of EAR and LAR (Fig. 2 A). EAR was typically a brief elevation of sRaw 15 min after 5% OVA provocation and LAR was typically observed 5 h later, consistent with an earlier report (Cieslewicz et al., J. Clin. Invest. (1999)104:301-308). Both EAR and LAR have magnitudes between 5 to 30 fold over the baseline. The severe airway response during the peak of LAR was generally evident as animals showed obvious labored breathing. Since inhibition of C5 can be achieved by the administration of anti-C5 mAb, we sought to dissect the critical involvement of C5 and its activated components at three critical points (Fig. 2 B) during critical stages of the disease. The three critical checkpoints are: 1) initiation of airway inflammation, one of the two hallmarks of asthma's pathogenesis; 2) development of AHR to nonspecific stimuli, another hallmark of asthma; and 3) sustainment of an on-going airway response after allergen provocation. Both checkpoint 2 and checkpoint 3 are designed to evaluate the contribution of C5 in subjects that had experienced severe airway response previously (Fig. 2 B) and had established airway inflammation. Checkpoint 3 studies were conducted during an on-going airway response after allergen provocation by administering treatment via either an i.v. or aerosol route during the EAR. Functions of lower airways and quantification of airway inflammation were evaluated during the peak of LAR, 5 h after 5% OVA provocation.

Checkpoint 1: Involvement of C5 in the initiation of airway inflammation

C5 inhibition at checkpoint 1, at the time of repeat aerosol sensitization of 1% OVA, had no significant impact on serum levels of OVA-specific IgE or IgG on day 32 (Fig. 3 A).

Samsung Bioepis Exhibit No. 1002
Page 8170

Corticosteroid treated mice also had elevated levels of OVA-specific IgE and IgG, that may have been generated long before the corticosteroid or anti-C5 mAb was administered on day 25. Sham treated mice had a negligible level of OVA specific IgG or IgE. As a result of repeated administration (i.p.) of anti-C5 mAb, approximately 80% of C5b-9 mediated hemolysis was inhibited on day 32 (21.1%±4) as compared to the normal hemolytic activity seen in control mAb and corticosteroid treated mice (92.7%±6.8). Two key functions of lower airways were examined by tracheal cannulation at 5 h after 5% OVA provocation. The increase in lung resistance (RL) is commonly associated with significant obstruction of lower airways (Fig. 3 B) and a decrease in the dynamic lung compliance (Cdyn) is associated with the loss of lung elasticity, an important characteristic of the return to normal volume after pressure changes seen in a normal lung (Fig. 3 C). The significantly increased RL and reduced Cdyn seen in control mAb-treated mice indicated an increased airway obstruction (Figs. 3 B and C). Sham-mice provided normal ranges of RL and Cdyn at 5 h after exposure to aerosol PBS solution.

Correlating to its potent anti-inflammatory and anti-asthmatic activities, corticosteroid treatment at checkpoint 1 significantly blocked the increase of RL and prevented the loss of Cdyn. Similarly, C5 inhibition at checkpoint 1 markedly reduced the increase of RL 5 h after allergen provocation with an RL of 1.66 cm H2O/ml/second ±0.2, compared to an RL of 2.17 cm H_2O/ml/second ±0.33 seen in control mAb treated animals (Fig. 3 B). C5 inhibition also slightly reduced the loss of Cdyn (Fig. 3 C). Double blind histological analysis (Fig. 3 D) of lung tissue confirmed data from the function analysis and demonstrated that control mAb-treated BALB/c mice had severe perivascular and peribronchial infiltration of inflammatory cells (Fig. 3 F). A significant reduction of histology score was observed in corticosteroid treated mice (Fig. 3 G) while less impressive reduction was observed in anti-C5 mAb-treated mice (Fig. 3 H). Similar to severe allergic asthma in humans, eosinophils were the predominant inflammatory cells (more than 50%) in the perivascular and peribronchial lesions along the airways (Figs. 3 F-H).

Checkpoint 2: Contribution of C5 in the development of AHR in animals with established airway inflammation

In addition to airway inflammation, AHR to non-specific stimuli is another hallmark of asthma. AHR was evaluated as changes of RL and Cdyn expressed as a percentage of baseline in response to increasing doses of aerosol methacholine (Mch) challenges (Figs. 4 A and B).

Samsung Bioepis Exhibit No. 1002
Page 8171

Control mAb-treated BALB/c mice had significant increases of RL and significant losses of Cdyn during the course of aerosol Mch challenges, while sham-mice responded only modestly to aerosol Mch challenges. Two of eight among control mAb-treated mice died during high doses of aerosol Mch challenge due to severe respiratory stress. In contrast, C5 inhibition prevented dramatic increases of RL and the reduction of Cdyn during the course of aerosol Mch challenges, similar to those animals treated with corticosteroid (Figs. 4 A and B). The loss of Cdyn and increase of RL during the course of aerosol Mch challenges compared to sham-mice were probably due to the presence of established airway inflammation in these animals. There were no noticeable differences in the degree of airway inflammation among the three treated cohorts. Anti-C5 mAb treated mice had approximately 45% of normal hemolytic activity on day 35.

In order to compare our results of C5 inhibition at checkpoint 2 with data from a previous report (Karp et al., Nat. Immunol. (2000) 1:221-226), the presence or absence of native intrinsic AHR was examined in some strains of normal C5d mice as previously reported (De Sanctis et al., Am. J. Respir. Crit. Care Med. (1997) 156:S82-S88; Levitt et al., FASEB J. (1998) 2:2605-2608). Non-immunized C5d AKR/J mice have dramatic airway responses to increasing doses of aerosol Mch challenges with significant increases of RL over baseline (84.2%±16.7 with 20 puffs Mch) and a significant reduction of Cdyn under baseline (40% ±5.4 with 20 puffs of Mch) whereas C5d SWR/J and B10D2oSn mice have minimal changes of RL and Cdyn, indistinguishable from C5 sufficient (C5s) BALB/c mice and C57BL/6 mice, with no more than a maximal change of 15% of either RL or Cdyn away from baseline (n=3 for each strain, data not shown). Next, C5d B10D2oSn mice, which do not have intrinsic AHR, were immunized and challenged with aerosol Mch in the identical manner as C5s BALB/c mice in Figs. 4 A and B. Subgroups of sensitized C5d animals were also given either control or anti-C5 mAb treatment on day 33. C5d B10D2oSn mice developed a similar degree of airway inflammation as C5s mice, with an average histology score of 1.91 ± 0.37 (n=6) compared to a histology score of 2.25 ± 0.25 in C5s BALB/c mice (n=4). No noticeable histological difference was observed between anti-C5 mAb and control mAb treated C5d mice. In contrast to a previous report (Karp et al., *supra*), increasing doses of aerosol Mch challenges did not induce significant changes of lower airway functions in OVA immunized B10D2.oSn mice (Figs. 4 C and D). Treatment with either anti-C5 mAb or control mAb on day 33 had no impact on the development of AHR. The data from both mAb treated cohorts were pooled together as an mAb treated cohort (Figs. 4 C and D).

Samsung Bioepis Exhibit No. 1002
Page 8172

Furthermore, reconstitution of OVA immunized B10D2.oSn mice with recombinant mouse C5a (rmC5a) completely restored AHR in response to aerosol Mch challenge as indicated by the significant increase of RL and reduction of Cdyn (Figs. 4 C and D).

Reconstitution of rmC5a was not sufficient for the development of AHR since sham-B10D2.oSn mice reconstituted with rmC5a did not have significant changes of lower airway functions during Mch challenge (Figs. 4 C and D). Analysis of serum samples harvested from each mouse after Mch challenges confirmed the C5 deficient status of B10D2.oSn mice with average hemolytic activity of 8.1% ± 0.9 compared to 108%±3.8 of C5s BALB/c mice. Furthermore, random tail samples (n=2) from each lot of C5s and C5d animals were analyzed to confirm the C5 genotype status.

Checkpoint 3: Contribution of C5 during an on-going airway response

The role of C5 in sustaining an on-going airway response was also examined. Intervention was given during the peak of EAR, which was monitored by DCP at 15 min after aerosol exposure to 5% OVA on day 35. All control mAb-treated BALB/c mice developed severe LAR with significant increases of RL and reductions of Cdyn 5 h after allergen provocation (Figs. 5 A and B).

Administration of treatment through i.v. injection during EAR was selected to achieve rapid systemic C5 inhibition, which completely blocked the development of LAR with minimal increase of RL 5 h after 5% OVA provocation (Fig. 5 A) and prevented much of the loss of lung elasticity indicated by the minimal reduction of Cdyn (Fig. 5 B). Interestingly, corticosteroid treatment did not eliminate the development of LAR with significant elevation of RL and reduction of Cdyn at 5 h.

Although no immediate impacts (within 5 h) on lower airway functions were observed after i.v. corticosteroid treatment (Fig. 5 A), this treatment significantly modulated the intrapulmonary IL-13 (Fig. 7 B) and significantly improved lower airway functions 24 h later. Double blind histologic analysis of lung tissue showed comparable levels of perivascular and peribronchial infiltration of inflammatory cells from the animals 5 h after i.v. intervention with either corticosteroid, anti-C5 mAb or control mAb, similar to the histological sample shown in Fig. 3 F.

Samsung Bioepis Exhibit No. 1002
Page 8173

Effect of C5 Inhibition on the Migration of Inflammatory Cells

The migration of inflammatory cells from airway tissue inflammation into bronchial lumen as enumerated was also examined by bronchial alveolar lavage fluid (BALF) WBC analysis. There was a significant reduction of total BALF inflammatory cells in anti-C5 mAb-treated BALB/c mice compared to control mAb-treated mice at checkpoint 1 (Fig. 6 A). The blockade on the migration of inflammatory cells by C5 inhibition exceeded the degree of improved lower airway function (Figs. 3 B and C) and reduced tissue inflammation (Fig. 3 D). Similarly, when BALF was harvested at checkpoint 3 (Fig. 6 B), C5 inhibition again had significant blockade on the migration of inflammatory cells into bronchial lumen, with significantly lower BALF WBC counts in anti-C5 treated animals than in animals treated with either corticosteroid or control mAb. This result is in dramatic contrast to the prominent presence of perivascular and peribronchial infiltration of inflammatory cells in lung tissue harvested before allergen provocation on day 35. The significant blockade on the migration of inflammatory cells by C5 inhibition correlated with the significantly enhanced efficacy of C5 inhibition over corticosteroid therapy at checkpoint 3 (Figs. 5 A and B).

Sham mice provided the baseline level of BALF WBC counts, which are primarily normal alveolar macrophages (82.3%±10.3) in WBC differential analysis (Fig. 6 C). Eosinophils constituted more than 45% of BALF inflammatory cells from all OVA immunized animals, regardless of the therapeutic intervention. There was a significant reduction in the percentage of neutrophils recovered in BALF from the animals treated at checkpoint 3 with either corticosteroid (4.5%±2.8) or anti-C5 mAb (6.3%±3.3) versus control mAb treated mice (18.3%±4.4). No significant reductions occurred in the percentages of eosinophils or lymphocytes in BALF samples from the animals given intervention at checkpoint 3 (Fig. 6 C).

Effect of C5 Inhibition on Intrapulmonary Th1/Th2 Cytokine Profile and Inflammatory Mediators

BALF harvested from control mAb treated mice had markedly increased levels of IL-5 and IL-13 (Figs. 7 A and B) as compared to sham mice. Corticosteroid treatment markedly reduced the BALF levels of IL-5 and IL-13, however, this did not translate into an immediate improvement of lower airway function (Figs. 5 A and B) during the 5 h period of treatment. The dramatic improvement of lower airway functions (Figs. 5 A and B) seen after C5 inhibition at

Samsung Bioepis Exhibit No. 1002
Page 8174

checkpoint 3 did not correlate with any reduction of BALF level of IL-5 and IL-13. The statistical difference of the BALF level of IL-13 between anti-C5- and corticosteroid-treated mice equals a p value of 0.051 C5 inhibition or corticosteroid therapy at checkpoint 3 did not influence the BALF level of histamine (Fig. 7 C). This was expected since histamine had probably already been released into the airway lumen upon the engagement of IgE receptors of mast cells by aerosol allergen provocation and was responsible for the appearance of EAR when therapeutic intervention was given. Eosinophils, the most prominent inflammatory cells along lower airways (Figs. 3 F-H), are probably responsible for the production of eotaxin, RANTES and TGF-β (Figs. 7 D-F) in asthmatic individuals. Control mAb-treated animals had significantly elevated BALF levels of eotaxin (Fig. 7 D), RANTES (Fig. 7 E), or activated TGF-β (Fig. 7 F), compared to negligible or non-detectable levels of these mediators in sham-mice. In contrast, C5 inhibition had a marked impact on BALF levels of eotaxin, RANTES, and activated TGF-β when given either at checkpoint 1 (Figs. , D and E) or checkpoint 3 (Fig. 7 F). Corticosteroid treatment only reduced the BALF level of TGF-β when given at checkpoint 3 but had no obvious impact on the production and release of eotaxin or RANTES when given at checkpoint 1.

Neutrophils are commonly believed to be responsible for the production of bronchial TNF-α and proMMP9. C5 inhibition significantly reduced BALF levels of TNF-α (Fig. 7 G) when given at checkpoint 1, significantly different from control mAb-treated mice. The bronchial level of proMMP9 was dramatically reduced and was significantly different from control mAb-treated and corticosteroid-treated mice when C5 inhibition was given at checkpoint 3 (Fig. 7 H).

Checkpoint 3: Contribution of Intrapulmonary Activation Of C5 During An On-Going Airway Response

The massive migration of inflammatory cells from airway tissue inflammation into bronchial lumen probably results from powerful chemotactic forces from the epithelial mucosa of the airway. Thus, C5 may be activated intrapulmonarily during an airway response to allergen provocation. The functions of lower airways and parameters of inflammation were evaluated after blocking the intrapulmonary activation of C5. The potential impact at checkpoint 3 by inhibitory receptors, such as FcγRIIB (Katz, Curr. Opin. Immunol. (2002) 14:698-704; Ravetch

Samsung Bioepis Exhibit No. 1002
Page 8175

et al., Annu. Rev. Immunol. (2001) 19:275-90), were also evaluated, as the result of interaction between intrapulmonary C5 and anti-C5 mAb and the subsequent formation of immune complexes (IC).

Effect of intrapulmonary C5 inhibition with anti-C5 mAb was directly compared to anti-C5 Fab in order to achieve this goal. Furthermore, the efficacy of an anti-C5aR serum IgG (sIgG) was evaluated in order to dissect the role of intrapulmonary C5a versus C5b-9 at this checkpoint. As demonstrated in Figs. 8 A and B, control mAb-treated animals developed severe LAR with significant increases of RL and significant reductions of Cdyn, in significant contrast to the normal RL and Cdyn seen in sham-mice. Aerosol administration of corticosteroid blocked the development of LAR as evidenced by the reduction of RL with corresponding preservation of Cdyn. Blocking intrapulmonary C5 activation with aerosol administration of either anti-C5 mAb or anti-C5 Fab during the peak of EAR also prevented the development of LAR with minimal increase of RL and minimal reduction of Cdyn (Figs. 8 A and B). RL and Cdyn of both anti-C5 treated cohorts were significantly different from control mAb treated animals. Blocking the binding of intrapulmonary C5a to its receptors with an anti-C5aR sIgG also prevented the development of LAR with minimal increase of RL and minimal reduction of Cdyn similar to animals treated with anti-C5 mAb. There was no statistical difference between the three cohorts treated with either anti-C5 mAb, anti-C5 Fab, or anti-C5aR sIgG (Figs. 8 A and B).

When the parameters of intrapulmonary inflammatory activities were examined, such as the migration of inflammatory cells (Fig. 8 C) and the BALF level of proMMP9 (Fig. 8 D), significant differences were found between blocking intrapulmonary activation of C5 versus blocking the binding of C5a to its C5aR. Aerosol administration of either anti-C5 mAb or its Fab fragment during EAR significantly blocked both the migration of inflammatory cells into bronchial airway lumen and the elevation of BALF level of proMMP9, whereas blocking the engagement of C5aR had no significant impact on these two key inflammatory parameters similar to the animals treated with control mAb (Figs. 8 C and D). Consistent with its ability to block the development of LAR (Figs. 8 A and B), aerosol administration of corticosteroid also ameliorated intrapulmonary inflammatory activities with marked reduction of the migration of inflammatory cells and the production of proMMP9 (Figs. 8 C and D) indicating the importance of inflammatory activities at the epithelial side of the bronchial lumen. Aerosol administration

Samsung Bioepis Exhibit No. 1002
Page 8176

of anti-C5 mAb, anti-C5 Fab or anti-C5aR sIgG did not have any impact on serum C5b-9 mediated hemolytic activity.

Combination Therapy:

As demonstrated in Fig. 9, animals treated with a non-specific control antibody had significant increases of lung resistance during the course of Mch challenges. In this study, two animals challenged with high doses of Mch died due to severe respiratory stress. In contrast, both the steroid and anti-C5 treated animals have only moderate increases of lung resistance, higher than normal sham immunized animals but significantly lower than control placebo treated mice. Combined treatment with both anti-C5 antibody and steroid significantly further reduced the increases of lung resistance during the course of Mch challenges. Based on this result, both steroid and C5 inhibitor can prevent the development of airway hyper-responsiveness to non-specific aerosol stimuli in asthmatic individuals with established airway inflammation.

Nebulization Formulations

Examples of formulations suitable for nebulization of an antibody are shown in Fig. 10. Fig. 11 further demonstrates the effectiveness of nebulization treatment. The results shown in Fig. 11 were obtained following a study that employed the same protocol as the Checkpoint 2 study described herein, except that the treatment here was delivered by nebulization on day 32 instead of i.p. For anti-C5 antibody, 3 mg/ml of BB5.1 was subject to 10 min nebulization, and 2 mg/ml steroid was also subject to 10 min nebulization. For the combination therapy, the final concentrations subjected to 10 min nebulization were: 3 mg/ml for BB5.1 and 2 mg/ml for the steroid.

Fig. 12 further shows that eculizumab in Formulation 1 (Fig. 10) at 30 mg/ml can be effectively and efficiently nebulized by a conventional nebulizer such as the Pari LC Jet Plus or a specialty nebulizer such as Sonik LDI. Fig. 12 shows that a majority of the particles after nebulization were less than 5 μM, which is suitable for deep lung delivery.

As shown in Fig. 13, eculizumab in Formulation 1 (Fig. 10) can be effectively and efficiently delivered by a conventional nebulizer, comparable to the aerosol characteristics of an existing pulmonary drug delivered by inhalation, Pulmozyme®, also using a conventional

Samsung Bioepis Exhibit No. 1002
Page 8177

nebulizer. Eculizumab in Formulation 1 (Fig. 10) can also be effectively and efficiently delivered by a specialty nebulizer, for example, Sonik LDI.

Using SDS-PAGE and HPLC analyses, Fig. 14 and Fig. 15 demonstrate that eculizumab can be effectively and efficiently delivered by nebulization. Eculizumab in a suitable formulation was nebulized using either the Pari-Jet Air nebulizer or the Sonik LDI nebulizer. The nebulized antibody, in a spray form, was collected from the mouth piece of the nebulizer (e.g., F1D2F), and different sizes of mouth pieces (e.g., F1D2C1-C5) were used. The SDS-PAGE and HPLC analyses demonstrated the integrity of the nebulized antibody through various sizes of the spray mouth piece, as shown by the purity of the samples analyzed.

References

All publications and patents mentioned herein, are hereby incorporated by reference in their entirety as if each individual publication or patent was specifically and individually indicated to be incorporated by reference.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the application described herein. Such equivalents are intended to be encompassed by the following claims.

Samsung Bioepis Exhibit No. 1002
Page 8178

We claim:

1. A method for preventing or treating a pulmonary condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade and an additional active agent.
2. The method of claim 1, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD) interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.
3. The method of claim 1, wherein the subject is a human.
4. The method of claim 1, wherein the antibody is specific to C5.
5. The method of claim 1, wherein the antibody is specific to C5 convertase.
6. The method of claim 1, wherein the antibody is specific to C5a or C5b-9.
7. The method of claim 1, wherein the antibody is eculizumab or pexelizumab.
8. The method of claim 1, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.
9. The method of claim 7, wherein the mutated Fc portion does not activate complement.
10. The method of claim 1, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.
11. The method of claim 1, wherein the additional active agent is a corticosteroid.
12. The method of claim 1, wherein the corticosteroid is dexamethasone.
13. The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by the same delivery method or route.
14. The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by different delivery methods or routes.

Samsung Bioepis Exhibit No. 1002
Page 8179

15. An aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary condition in a subject.

16. The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. The aerosol composition of claim 15 further comprising an additional active agent.

18. A biopharmaceutical package comprising: a) an antibody that inhibits activation of the complement cascade; and b) a nebulizer, wherein the package is suitable for preventing or treating a pulmonary condition in a subject.

19. The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. The biopharmaceutical package of claim 19 further comprising instructions for use.

21. The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. A pulmonary drug delivery kit comprising: a) an inhaler; and b) an antibody formulated for inhalation delivery.

23. The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. The kit of claim 23, whereon the metered dose inhaler is a pressurized metered dose inhaler.

25. The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. The kit of claim 22, wherein said inhaler is a nebulizer.

27. The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

28. The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. A pulmonary drug delivery kit comprising a) an anti C5 antibody composition comprising 1 mg/ml to 200 mg/ml; and b) a label indicating administration by nebulization.

Abstract

The present application relates to methods and compositions employing an antibody that inhibits activation of the complement system and can be used to prevent or treat a pulmonary disease or condition.

Samsung Bioepis Exhibit No. 1002
Page 8181

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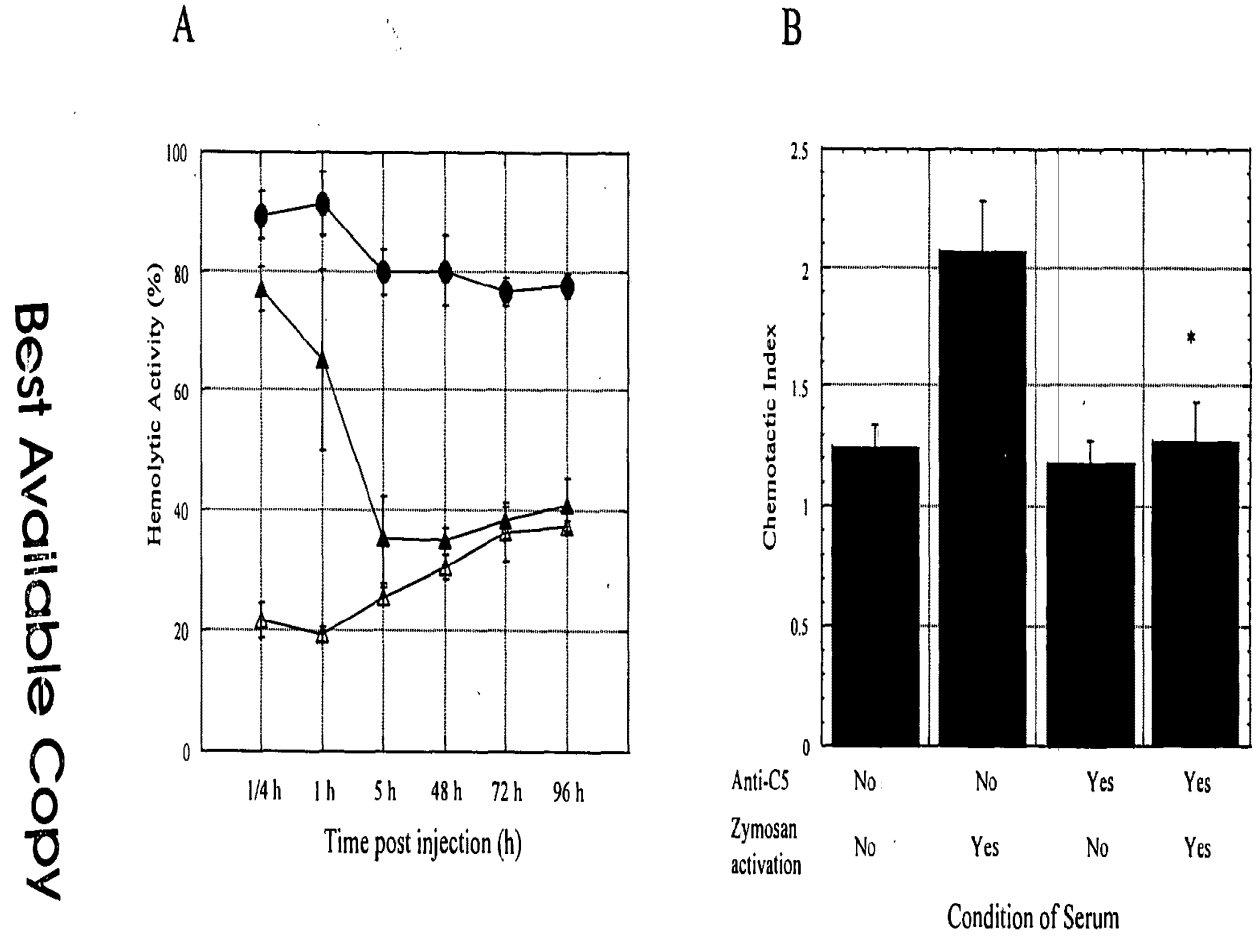
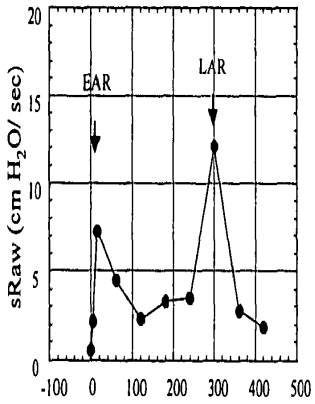


Fig. 1

A



B

Induction of disease (days post initial immunization on day 0)

Day 28-30: Initiation of airway inflammation with daily aerosol sensitization of 1% OVA → Day 32: Induction 1st severe airway response with aerosol provocation of 5% OVA → Day 33: Untreated mice recover from 1st airway response of day 32. Animals have established airway inflammation → Day 35: Induction of 2nd severe airway response. Untreated mice were aerosol challenged again with 5% OVA.

Checkpoint 1: Initiation of airway inflammation (C5 inhibition on days 25, 29 & 31) → Evaluation of the development of 1st airway response to allergen provocation & the degree of airway inflammation

Checkpoint 2: Development of AHR in mice with established airway inflammation (C5 inhibition on day 33) → Evaluation of AHR to aerosol methacholine challenges on day 35

Checkpoint 3: During an ongoing airway response (C5 inhibition during EAR) → Evaluation of functions of lower airways during LAR & analysis of BALF

52

Fig. 2

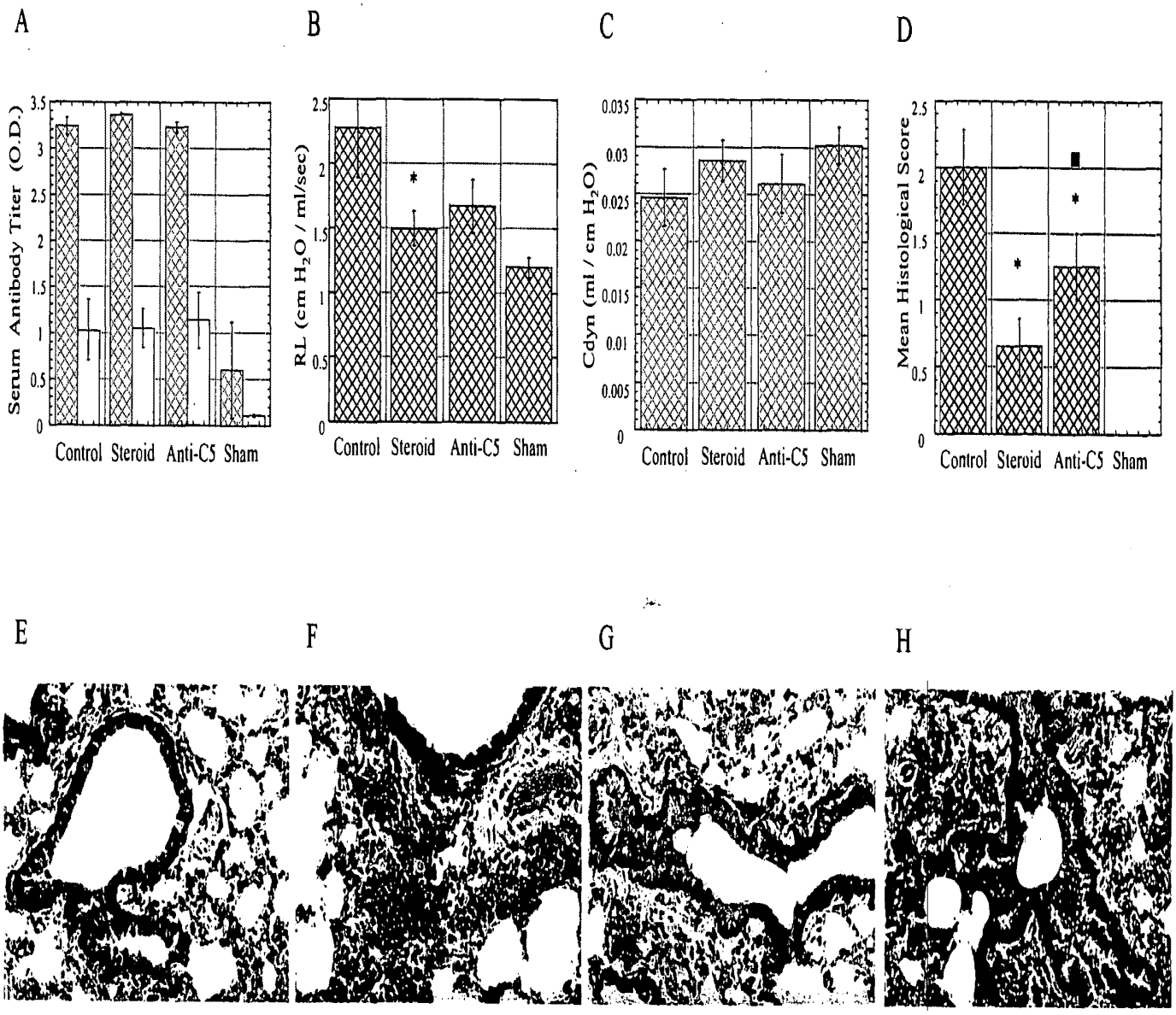
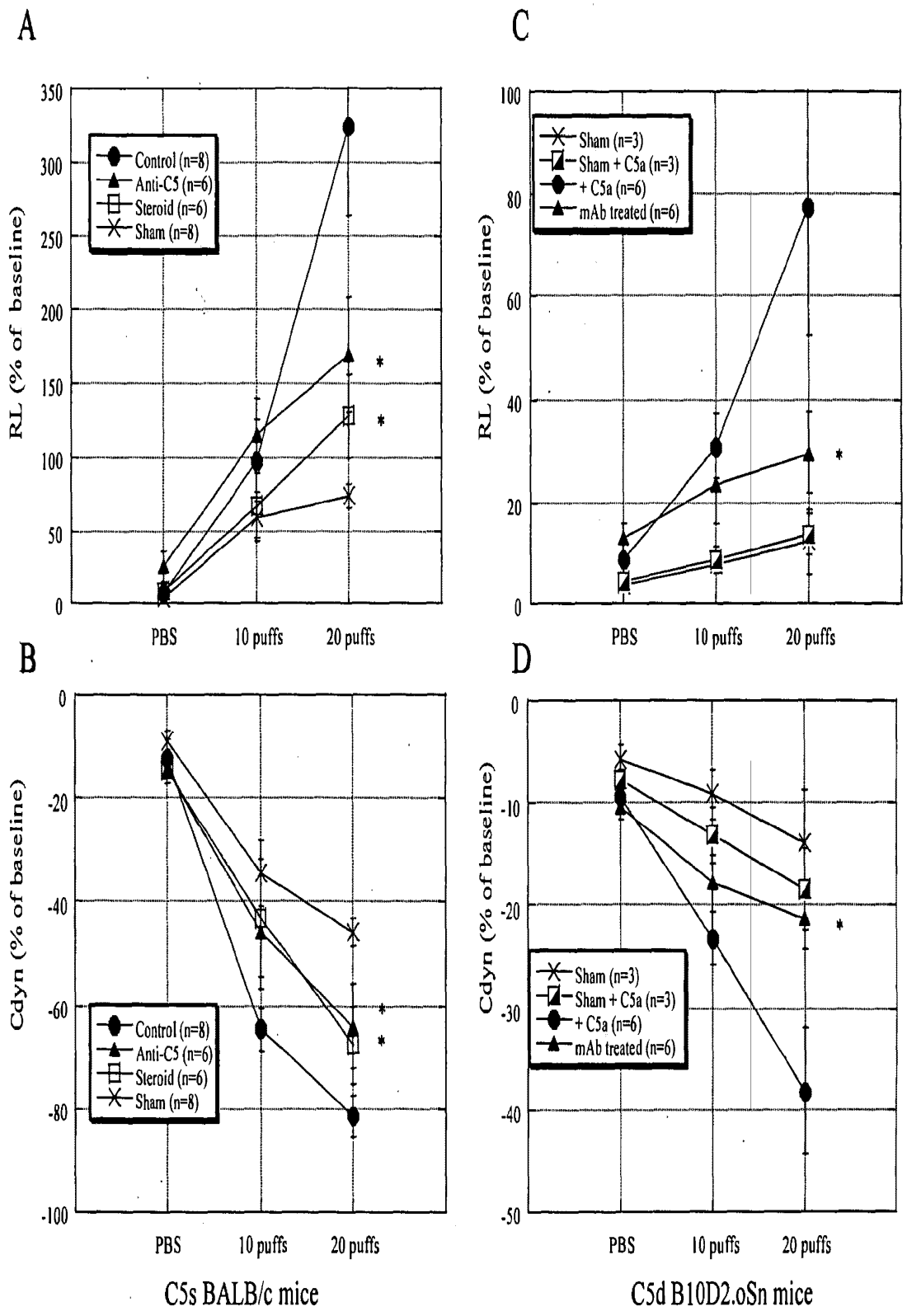

A
B
C
D
Serum Antibody Titer (O.D.)
RL (cm H2O / ml/sec)
Cdyn (ml / cm H2O)
Mean Histological Score
Control Steroid Anti-C5 Sham
E
F
G
H

Fig. 3


A
C
B
D
RL (% of baseline)
Cdyn (% of baseline)
Control (n=8)
Anti-C5 (n=6)
Steroid (n=6)
Sham (n=8)
Sham (n=3)
Sham + C5a (n=3)
+ C5a (n=6)
mAb treated (n=6)
PBS
10 puffs
20 puffs
C5s BALB/c mice
C5d B10D2.oSn mice

54

Fig. 4

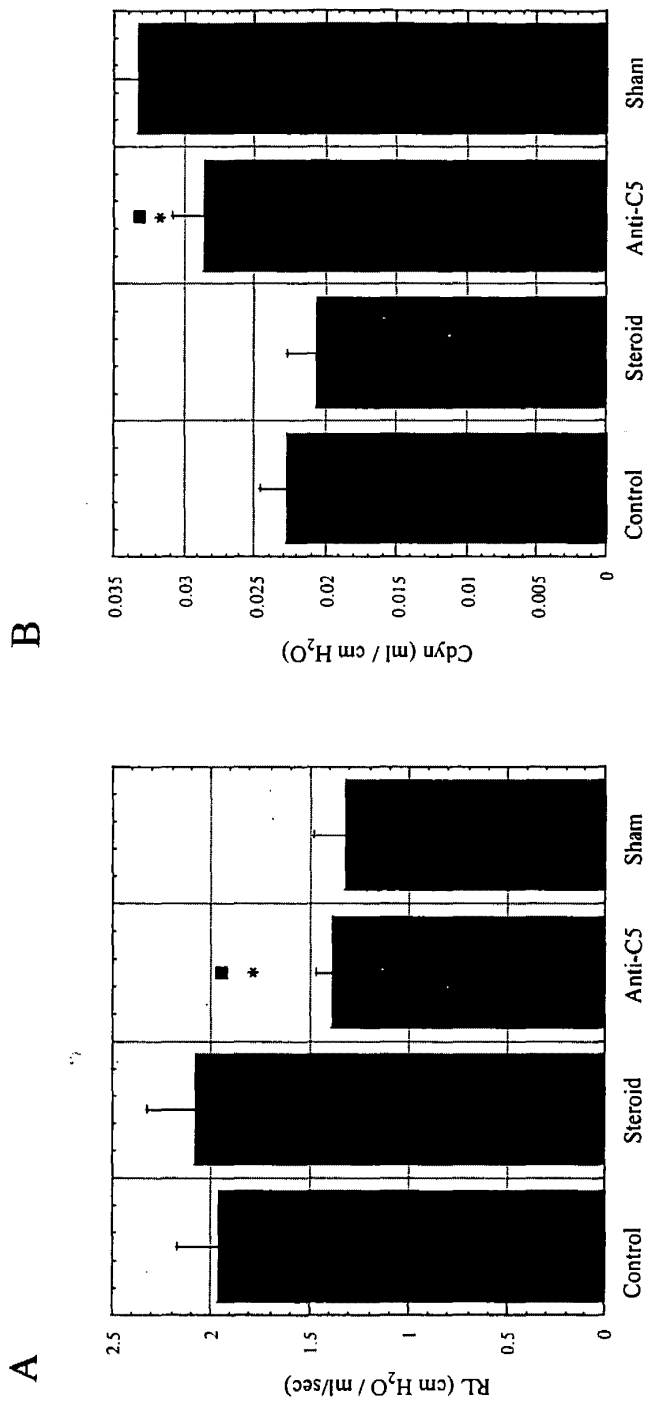
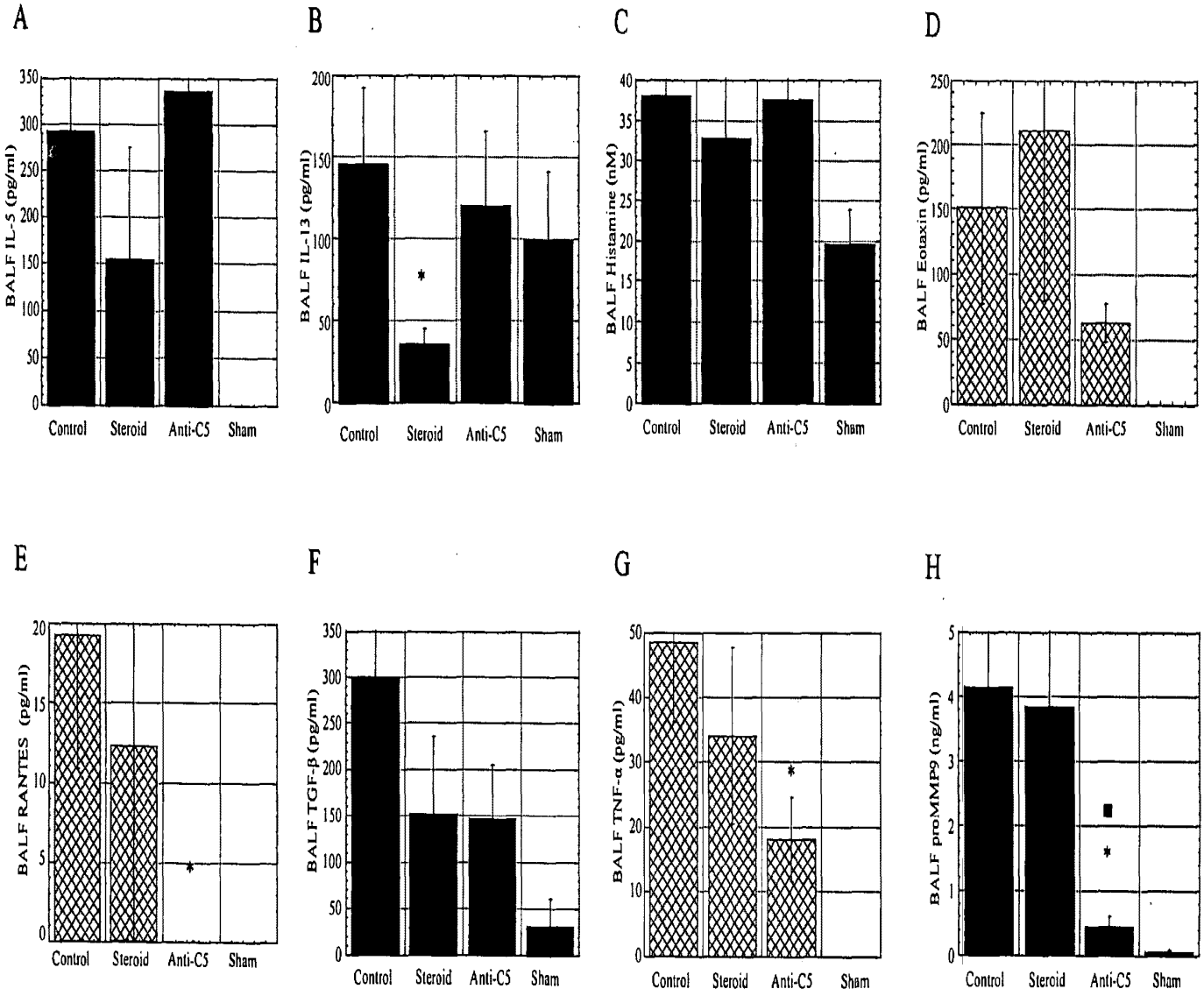


Fig. 5

Samsung Bioepis Exhibit No. 1002
Page 8186

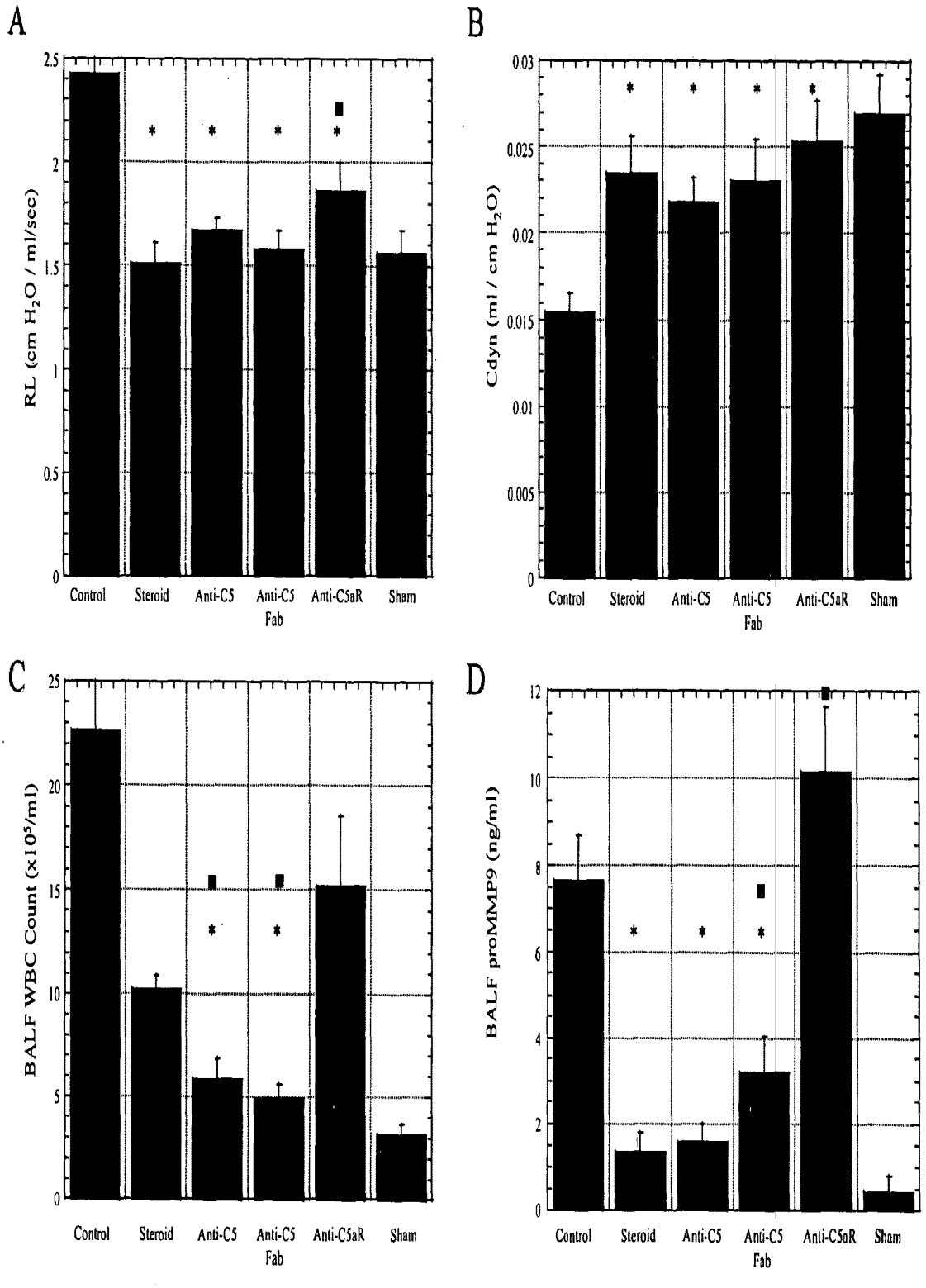
Fig. 6

Samsung Bioepis Exhibit No. 1002
Page 8187


A
BALF IL-5 (pg/ml)
350
300
250
200
150
100
50
0
Control
Steroid
Anti-C5
Sham
B
BALF IL-13 (pg/ml)
200
150
100
50
0
*
Control
Steroid
Anti-C5
Sham
C
BALF Histamine (nM)
40
35
30
25
20
15
10
5
0
Control
Steroid
Anti-C5
Sham
D
BALF Eotaxin (pg/ml)
250
200
150
100
50
0
Control
Steroid
Anti-C5
Sham
E
BALF RANTES (pg/ml)
20
15
10
5
0
*
Control
Steroid
Anti-C5
Sham
F
BALF TGF-β (pg/ml)
350
300
250
200
150
100
50
0
Control
Steroid
Anti-C5
Sham
G
BALF TNF-α (pg/ml)
50
40
30
20
10
0
*
Control
Steroid
Anti-C5
Sham
H
BALF proMMP9 (ng/ml)
5
4
3
2
1
0
*
Control
Steroid
Anti-C5
Sham

57

Fig. 7


A
RL (cm H_2O / ml/sec)
2.5
2
1.5
1
0.5
0
Control
Steroid
Anti-C5
Anti-C5 Fab
Anti-C5aR
Sham
B
Cdyn (ml / cm H_2O)
0.03
0.025
0.02
0.015
0.01
0.005
0
Control
Steroid
Anti-C5
Anti-C5 Fab
Anti-C5aR
Sham
C
BALF WBC Count (×10^5/ml)
25
20
15
10
5
0
Control
Steroid
Anti-C5
Anti-C5 Fab
Anti-C5aR
Sham
D
BALF proMMP9 (ng/ml)
12
10
8
6
4
2
0
Control
Steroid
Anti-C5
Anti-C5 Fab
Anti-C5aR
Sham

58

Fig. 8

Fig. 9
2 days after aerosol Treatment

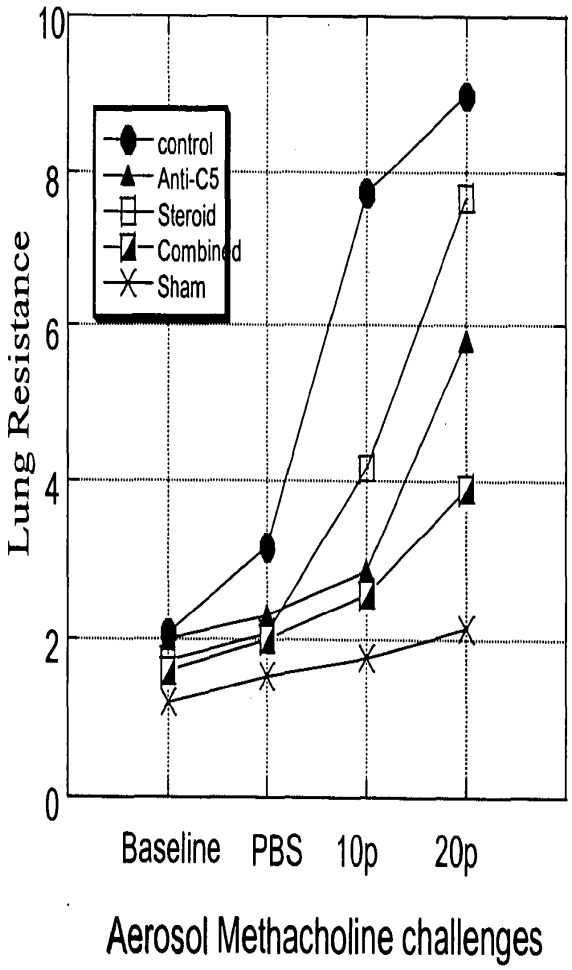


Fig. 10 Example of Suitable Formulations

Formulation 1

- Mab 1- 30 mg/ml
- 10 mM sodium phosphate
- 150 mM NaCl
- pH 6-8
- 0.001 to 0.02% Tween 80 v/v
- physiologic osmolality

Formulation 2

- 20mM Histidine
- 50mM Glycine
- 3% (w/v) Sorbital
- 1.5% (w/v) Mannitol
- pH 6-8
- 0.001 to 0.02% Tween 80
- 40mg/ml-200mg/ml
- physiologic osmolality

Samsung Bioepis Exhibit No. 1002
Page 8191

Fig. 11 Mch challenge two days after nebulization treatment

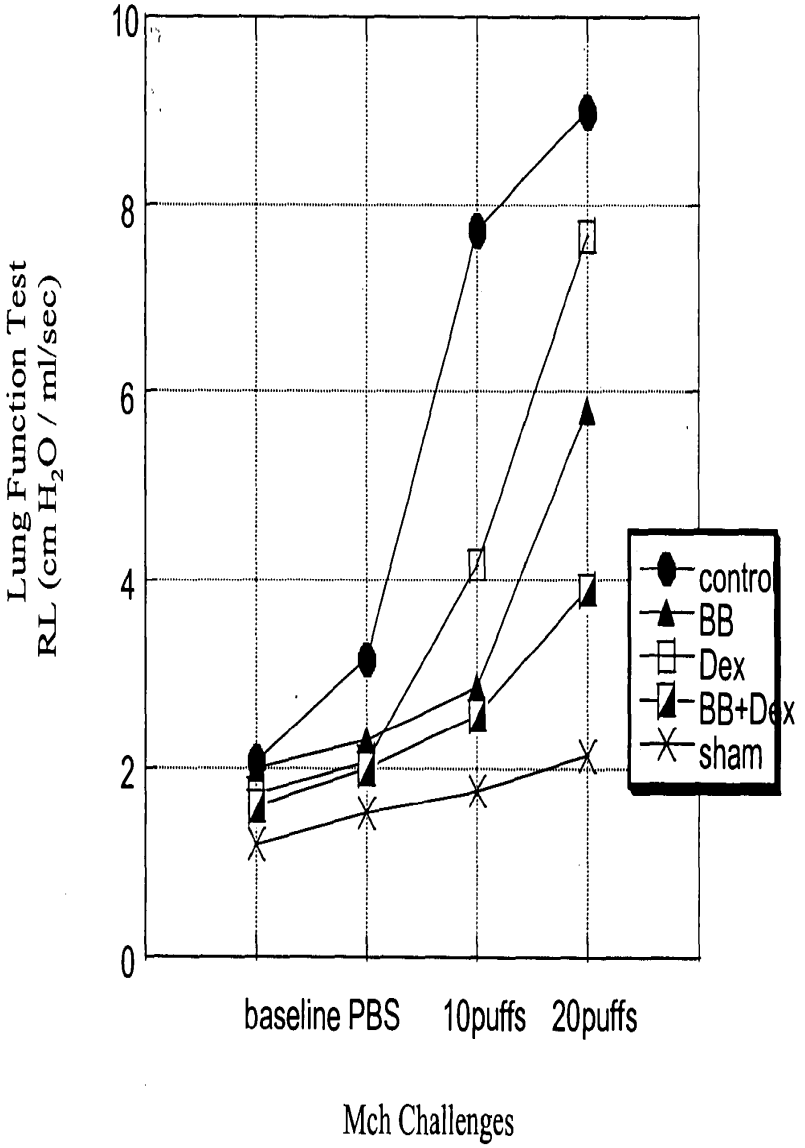
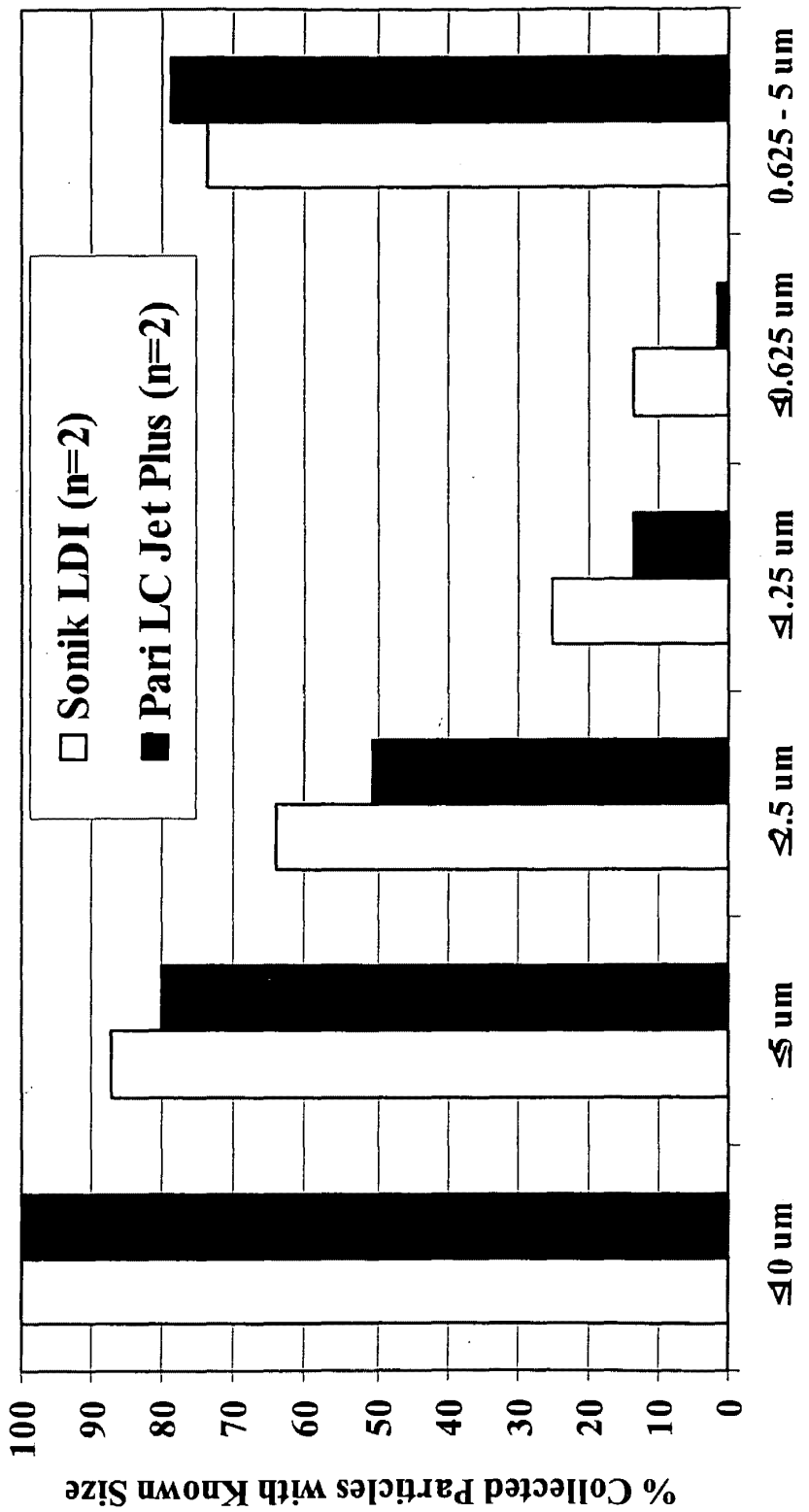


Fig. 12 Particle Size Distribution



Samsung Bioepis Exhibit No. 1002
Page 8193

Fig. 13 Nebulization and Delivery Efficiency

	Sonik LDI Eculizumab	Pari LC Jet Plus Eculizumab	Pari LC Jet Plus Pulmozyme
Respirable fraction (%)	44.91 – 59.63	71.88 – 74.21	47.8 (1 – 5 µm)
Nebulizer efficiency (%)	11.24 – 19.91	42.51 – 52.46	51.4
Delivery (%)	6.70 – 8.94	31.55 – 37.71	24.6 (1 – 5 µm)

Respirable fraction: the fraction of the droplets delivered to the exit from the mouth-piece in the range of 0.625 – 5 µm

Nebulizer efficiency: the fraction of the dose initially placed in the nebulizer that reaches the exit from the mouthpiece

Delivery: the fraction of the initial dose that reaches the exit from the mouthpiece in the size range of 0.625 – 5 µm

Samsung Bioepis Exhibit No. 1002
Page 8194

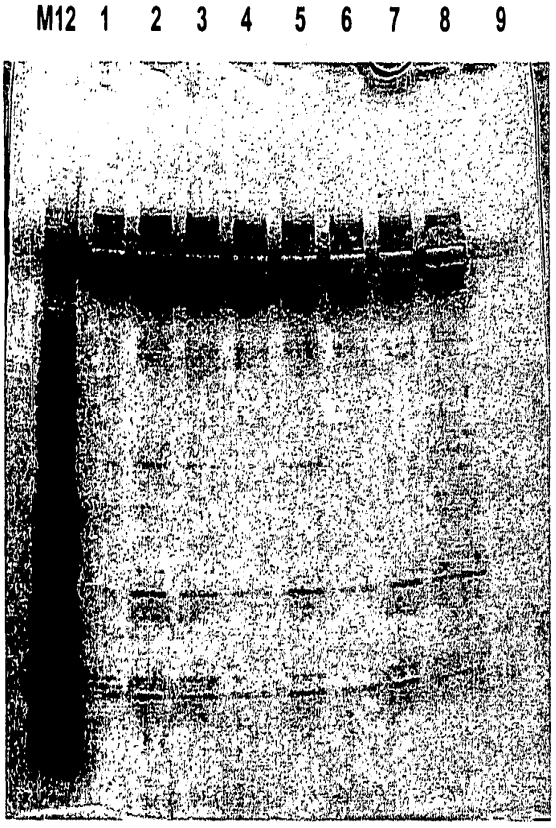
Fig. 14 Eculizumab Aerosolization

SDS-PAGE Analysis

4-20% TG, 1.5mm, 10 Well, Non Reduced

Reference NDG #1383 page 38-42

Pari-Jet Air



Lane	Sample	load (ug)	Purity(%)	% Peak Response
	MW marker	NA	NA	N/A
1	Ecu Ref Std	1.5	99.3	N/A
2	TR-CTRL-1 (30mg/mL)	1.5	99.1	102.7
3	16FEB05, F1D2C1	1.5	97.7	99.8
4	16FEB05, F1D2C2	1.5	98.5	98.6
5	16FEB05, F1D2C3	1.5	96.8	99.3
6	16FEB05, F1D2C4	1.5	94.3	94.1
7	16FEB05, F1D2C5	1.5	88.9	92.3
8	16FEB05, F1D2F	1.5	N/A	N/A
9	Blank	N/A	N/A	N/A

Samsung Bioepis Exhibit No. 1002
Page 8195

Fig. 15 Eculizumab Aerosolization

SEC HPLC Purity Summaries

	15 Feb 05		16Feb 05	
	Sonik LDI	Pari Jet Air	Sonik LDI	Pari Jet Air
F1P1-W1	99.1	98.4	99.0	98.4
F1P2-W1	99.2	99.2	98.2	98.5
F1P3-W1	99.1	99.2	99.1	99.1
F1P4-W1	95.7	NA	93.8	n/a
F1C1-W1	[95.6]	97.5	94.2	97.7
F1C2-W1	[96.1]	98.2	95.8	98.5
F1C3-W1	[94.9]	96.9	[96.3]	96.8
F1C4-W1	[93.0]	94.0	95.2	94.3
F1C5-W1	[90.8]	89.3	[92.7]	88.9
[Peak responses < 90%]				

Samsung Bioepis Exhibit No. 1002
Page 8196

Fig. 16 Repeat Long Term Aerosol Administrations of Anti-C5 mAb Do Not Induce Airway Inflammation (2x per week for 10wks)



Sham Treated (PBS)

Control mAb

Ant-C5 (BB5.1)

Alexion

Samsung Bioepis Exhibit No. 1002
Page 8197

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Page 8198

MULTIPLE DEPENDENT CLAIM FEE CALCULATION SHEET

SERIAL NO 11/127,438 FILING DATE 4-13-05

APPLICANT(S)

CLAIMS

	AS FILED		AFTER 1ST AMENDMENT		AFTER 2ND AMENDMENT		*		*		*	
	IND	DEP	IND	DEP	IND	DEP	IND	DEP	IND	DEP	IND	DEP
1	1											
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TOTAL IND.	5											
TOTAL DEP.	26											
TOTAL CLAIMS	31											

	IND	DEP	IND	DEP	IND	DEP
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100						
TOTAL IND.						
TOTAL DEP.						
TOTAL CLAIMS						

*MAY BE USED FOR ADDITIONAL CLAIMS OR AMENDMENTS

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

05/13/2005 HLE333 00000042 181945 11127438

01 FC:1011	300.00	DA
02 FC:1111	500.00	DA
03 FC:1311	200.00	DA
04 FC:1201	400.00	DA
05 FC:1202	550.00	DA
06 FC:1203	360.00	DA

PTO-1556
(5/87)

*U.S. Government Printing Office: 2002 — 489-267/69033

Samsung Bioepis Exhibit No. 1002
Page 8200

PATENT APPLICATION FEE DETERMINATION RECORD

Effective December 8, 2004

11127438

CLAIMS AS FILED - PART I

	(Column 1)	(Column 2)
TOTAL CLAIMS	30	
FOR	NUMBER FILED	NUMBER EXTRA
TOTAL CHARGEABLE CLAIMS	31 minus 20=	* 11
INDEPENDENT CLAIMS	5 minus 3 =	* 2
MULTIPLE DEPENDENT CLAIM PRESENT		☑

* If the difference in column 1 is less than zero, enter "0" in column 2

SMALL ENTITY TYPE ☐		OR	OTHER THAN SMALL ENTITY	
RATE	FEE		RATE	FEE
BASIC FEE	150.00	OR	BASIC FEE	300.00
X$ 25=		OR	X$50=	550
X100=		OR	X200=	400
+180=		OR	+360=	360
TOTAL		OR	TOTAL	1610

CLAIMS AS AMENDED - PART II

AMENDMENT A		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
	Total	*	Minus	**	=
	Independent	*	Minus	***	=
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 25=		OR	X$50=	
X100=		OR	X200=	
+180=		OR	+360=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT B		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
	Total	*	Minus	**	=
	Independent	*	Minus	***	=
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 25=		OR	X$50=	
X100=		OR	X200=	
+180=		OR	+360=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

AMENDMENT C		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA
	Total	*	Minus	**	=
	Independent	*	Minus	***	=
	FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM				☐

RATE	ADDITIONAL FEE		RATE	ADDITIONAL FEE
X$ 25=		OR	X$50=	
X100=		OR	X200=	
+180=		OR	+360=	
TOTAL ADDIT. FEE		OR	TOTAL ADDIT. FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.
** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20."
*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3."
The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Application Data Sheet

Application Information

Application Type::	Regular
Subject Matter::	Utility
Suggested Group Art Unit::	N/A
CD-ROM or CD-R?::	None
Sequence submission?::	None
Computer Readable Form (CRF)?::	No
Title::	NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES
Attorney Docket Number::	ALEX-P01-001
Request for Early Publication?::	No
Request for Non-Publication?::	No
Total Drawing Sheets::	16
Small Entity?::	No
Petition included?::	No
Secrecy Order in Parent Appl.?::	No

Applicant Information

Applicant Authority Type::	Inventor
Primary Citizenship Country::	US
Status::	Full Capacity
Given Name::	Yi
Family Name::	Wang
City of Residence::	Orange
Country of Residence::	CT

Correspondence Information

Correspondence Customer Number::	28120

Representative Information

Representative Customer Number:: 28120

Domestic Priority Information

Application::	Continuity Type::	Parent Application::	Parent Filing Date::
This Application	Continuation-in-part of	10/655861	09/05/03
10/655861	An application claiming the benefit under 35 USC 119(e)	60/408571	09/06/02
10/655861	An application claiming the benefit under 35 USC 119(e)	60/469189	05/09/03

Foreign Priority Information

Assignee Information

Assignee name::	Alexion Pharmaceuticals, Inc.
Street of mailing address::	352 Knotter Drive
City of mailing address::	Cheshire
State or Province of mailing address::	CT
Postal or Zip Code of mailing address::	06410

ARTIFACT SHEET

Enter artifact number below. Artifact number is application number + artifact type code (see list below) + sequential letter (A, B, C ...). The first artifact folder for an artifact type receives the letter A, the second B, etc..
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11,127438 CA

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March 8, 2004

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APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/127,438	05/11/2005	Yi Wang	ALEX-P01-001

CONFIRMATION NO. 5259

28120
FISH & NEAVE IP GROUP
ROPES & GRAY LLP
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

FORMALITIES LETTER

OC000000016657938

Date Mailed: 08/01/2005

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

FILED UNDER 37 CFR 1.53(b)

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

- The oath or declaration is missing. *A properly signed oath or declaration in compliance with 37 CFR 1.63, identifying the application by the above Application Number and Filing Date, is required.*
  Note: If a petition under 37 CFR 1.47 is being filed, an oath or declaration in compliance with 37 CFR 1.63 signed by all available joint inventors, or if no inventor is available by a party with sufficient proprietary interest, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(f) of $130 for a non-small entity, must be submitted with the missing items identified in this letter.

SUMMARY OF FEES DUE:

Total additional fee(s) required for this application is **$130** for a Large Entity

- **$130** Late oath or declaration Surcharge.

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PART 3 - OFFICE COPY

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APPLICATION NUMBER	FILING OR 371 (c) DATE	FIRST NAMED APPLICANT	ATTORNEY DOCKET NUMBER
11/127,438	05/11/2005	Yi Wang	ALEX-P01-001

CONFIRMATION NO. 5259

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OC000000016657938

Date Mailed: 08/01/2005

NOTICE TO FILE MISSING PARTS OF NONPROVISIONAL APPLICATION

08/23/2005 HLE333 00000005 181945 11127438 **FILED UNDER 37 CFR 1.53(b)**

01 FC:2051 65.00 DA

Filing Date Granted

Items Required To Avoid Abandonment:

An application number and filing date have been accorded to this application. The item(s) indicated below, however, are missing. Applicant is given **TWO MONTHS** from the date of this Notice within which to file all required items and pay any fees required below to avoid abandonment. Extensions of time may be obtained by filing a petition accompanied by the extension fee under the provisions of 37 CFR 1.136(a).

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  Note: If a petition under 37 CFR 1.47 is being filed, an oath or declaration in compliance with 37 CFR 1.63 signed by all available joint inventors, or if no inventor is available by a party with sufficient proprietary interest, is required.
- To avoid abandonment, a late filing fee or oath or declaration surcharge as set forth in 37 CFR 1.16(f) of ~~$130~~ 65 for a ~~non~~-small entity, must be submitted with the missing items identified in this letter.

SUMMARY OF FEES DUE:

Total additional fee(s) required for this application is ~~$130~~ 65 Small for a ~~Large~~ Entity

- ~~$130~~ 65 Late oath or declaration Surcharge.

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PART 2 - COPY TO BE RETURNED WITH RESPONSE

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PTO/SB/17 (12-04v2)
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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

[x] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 65.00

Complete if Known	
Application Number	11/127438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Examiner Name	Not Yet Assigned
Art Unit	1614
Attorney Docket No.	ALEX-P01-001

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[x] Deposit Account Deposit Account Number: 18-1945 Deposit Account Name: Ropes & Gray LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[x] Charge fee(s) indicated below [ ] Charge fee(s) indicated below, **except for the filing fee**

[x] Charge any additional fee(s) or underpayment of fee(s) under 37 CFR 1.16 and 1.17 [x] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	50	25
Each independent claim over 3 (including Reissues)	200	100
Multiple dependent claims	360	180

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____ - =	_____ x	_____ =	_____

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	_____

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
_____ - =	_____ x	_____ =	_____

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
_____ - 100 =	_____ /50	_____ (round up to a whole number) x	_____ =	_____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	
Other (e.g., late filing surcharge): 2051 Surcharge-Late oath or declaration	65.00

SUBMITTED BY

Signature	Anita Varma	Registration No. (Attorney/Agent)	43,221	Telephone	(617) 951-7796
Name (Print/Type)	Anita Varma			Date	August 18, 2005

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: MS Missing Parts, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: August 18, 2005 Signature: Mary Jane DiPalma (Mary Jane DiPalma)

9797830.1

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Page 8209

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Dated: August 18, 2005 Signature: (Mary Jane DiPalma)

Docket No.: ALEX-P01-001
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127438

Filed: May 11, 2005

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Confirmation No.: 5259

Art Unit: 1614

Examiner: Not Yet Assigned

RESPONSE TO NOTICE TO FILE MISSING PARTS OF APPLICATION

MS Missing Parts
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the Notice to File Missing Parts of Application – Filing Date Granted mailed August 1, 2005 (copy enclosed), Applicant respectfully submits a Combined Declaration and Power of Attorney and payment of the surcharge for late filing of the oath or declaration.

Please charge our Deposit Account No. 18-1945 in the amount of $65.00 covering the fee set forth in 37 CFR 1.16(f). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALEX-P01-001. A duplicate copy of this paper is enclosed.

Dated: August 18, 2005

Respectfully submitted,

By _____
Anita Varma
Registration No.: 43,221
FISH & NEAVE IP GROUP
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

Samsung Bioepis Exhibit No. 1002
Page 8210

OIPE JC71
AUG 22 2005
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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

DECLARATION FOR PATENT APPLICATION

As the below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

the specification of which was filed on **May 11, 2005** as Application No. **11/127438**.

In the event that the filing date and/or Application No. are not entered above at the time I execute this document, and if such information is deemed necessary, I hereby authorize and request my attorneys/agent(s) at **Ropes & Gray LLP**, One International Place, Boston, Massachusetts 02110-2624, to insert above the filing date and/or Application No. of said application.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by an amendment, if any, specifically referred to herein.

I acknowledge the duty to disclose all information known to me that is material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56.

FOREIGN PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code § 119(a)-(d) of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

[x] no such foreign applications have been filed

[ ] such foreign application have been filed as follows:

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Application Number	Country	Date of Filing	Priority Claimed Under 35 USC 119
			___ Yes No ___
			___ Yes No ___
			___ Yes No ___

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Application Number	Country	Date of Filing

CLAIM FOR BENEFIT OF EARLIER U.S. PROVISIONAL APPLICATIONS

I hereby claim priority benefits under Title 35, United States Code §119(e), of any United States provisional patent application(s) listed below:

☐ no such U.S. provisional applications have been filed.

☒ such U.S. provisional application have been filed as follows:

Application Number	Date of Filing	Priority Claimed Under 35 USC 119
60/408571	September 6, 2002	x Yes No ___
60/469189	May 9, 2003	x Yes No ___
		___ Yes No ___

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of the United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose all information that is material to patentability in accordance with Title 37, Code of Federal Regulations, §1.56 which became available to me between the filing date of the prior application and the national or PCT international filing date of this application:

☐ no such U.S./PCT applications have been filed.

☒ such U.S./PCT application have been filed as follows:

Application Number	Relationship	Parent Application	Date of Filing
This Application	Continuation-in-part	10/655861	September 5, 2003

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint:

All practitioners at Customer Number 28120

all of Ropes & Gray LLP, One International Place, Boston, Massachusetts 02110-2624, jointly, and each of them severally, my attorneys at law/patent agent(s), with full power of substitution, delegation and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, and to transact all business in the U. S. Patent and Trademark Office connected therewith.

The undersigned hereby authorizes the U.S. attorney or agent named herein to accept and follow instructions from Alexion Pharmaceuticals, Inc. as to any action to be taken in the United States Patent and Trademark Office regarding this application without direct communication between the U.S. attorney or agent and the undersigned. In the event of a change in the persons from whom instructions may be taken, the U.S. attorney or agent named herein will be so notified by the undersigned.

Please mail all correspondence to Anita Varma, whose address is:

Ropes & Gray LLP
One International Place
Boston, Massachusetts 02110-2624

Please direct telephone calls to: Anita Varma at (617) 951-7796.

Please direct facsimiles to: (617) 951-7050.

Full name of sole or first inventor Yi Wang	
Sole or first inventor's signature	Date 7/20/05
Residence Orange, CT	
Citizenship US	
Mailing Address 270 Sylvan Valley Road Orange, CT 06477	

Full name of second inventor, if any	
Second inventor's signature	Date
Residence	
Citizenship	
Mailing Address	

Full name of third inventor, if any	
Third inventor's signature	Date
Residence	
Citizenship	
Mailing Address	

Full name of fourth inventor, if any	
Fourth inventor's signature	Date
Residence	
Citizenship	
Mailing Address	

PATENT APPLICATION SERIAL NO. _____

U.S. DEPARTMENT OF COMMERCE
PATENT AND TRADEMARK OFFICE
FEE RECORD SHEET

05/13/2005 HLE333 00000042 181945 11127438

01 FC:1011 300.00 DA
02 FC:1111 500.00 DA
03 FC:1311 200.00 DA
04 FC:1201 400.00 DA
05 FC:1202 550.00 DA
06 FC:1203 360.00 DA

Adjustment date: 09/21/2005 SDIRETA1
05/13/2005 HLE333 00000042 181945 11127438
01 FC:1011 300.00 CR
02 FC:1111 500.00 CR
03 FC:1311 200.00 CR
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05 FC:1202 550.00 CR
06 FC:1203 360.00 CR

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01 FC:2011 150.00 DA
02 FC:2111 250.00 DA
03 FC:2311 100.00 DA
04 FC:2202 275.00 DA
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06 FC:2203 180.00 DA

PTO-1556
(5/87)

*U.S. Government Printing Office: 2002 — 469-267/68009

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Page 8215

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JUL 14 2005
PATENT & TRADEMARK OFFICE

Dep Pref

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: 7/11/05 Signature: (Valerie J. Sarosky)

Docket No.: ALEX-P01-001 (PATENT)

US PATENT AND TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127438

Confirmation No.: Not yet provided

Filed: May 11, 2005

Group Art Unit: Not yet provided

For: NEBULIZATION MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: Not yet provided

REQUEST FOR REIMBURSEMENT

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

The above-referenced patent application qualifies for small entity status. Applicant's Attorney respectfully requests a reimbursement of $955.00, one-half of the total fees paid on May 11, 2005, to be deposited in Deposit Account No. 18.1945.

A copy of this letter is enclosed for accounting purposes.

Dated: July 11, 2005

Respectfully submitted,

By _____
Anita Varma
Registration No.: 43,221
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

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Page 8216

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FAX TRANSMISSION

DATE: January 5, 2006

PTO IDENTIFIER: **Application Number** 11/127438
Patent Number
Inventor: Yi Wang

MESSAGE TO: US Patent and Trademark Office
FAX NUMBER: (571) 273-8300

FROM: FISH & NEAVE IP GROUP
ROPES & GRAY LLP
Xuqiong Wu, Ph.D.
PHONE: (617) 951-7728

Attorney Dkt. #: ALXN-P02-102

PAGES (Including Cover Sheet): 2

CONTENTS:
Change Of Attorney Docket Number (1 page)
This Facsimile Cover Sheet (1 page)

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One International Place, Boston, Massachusetts 02110-2624
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PAGE 1/2 * RCVD AT 1/5/2006 6:26:47 PM [Eastern Standard Time] * SVR:USPTO-EFXRF-6/28 * DNIS:2738300 * CSID:617 951 7050 * DURATION (mm-ss):00-42

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Page 8217

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Dated: January 5, 2006 Signature: (Mary Jane Palme)

Docket No.: ALXN-P02-102 (PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Wang Yi

Confirmation No.: 5259

Application No.: 11/127438

Group Art Unit: 1644

Filed: May 11, 2005

Examiner: Vandervegt, Francois P.

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

CHANGE OF ATTORNEY DOCKET NUMBER

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Please note that the Attorney Docket Number has been changed from ALEX-P01-001 to ALXN-P02-102. Please reference **ALXN-P02-102** on all future correspondence.

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 18-1945, under Order No. ALXN-P02-102 from which the undersigned is authorized to draw.

Dated: January 5, 2006

Respectfully submitted,

By
Xuqiong Wu, Ph.D.
Registration No.: 55,745
FISH & NEAVE IP GROUP
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624

(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

Samsung Bioepis Exhibit No. 1002
Page 8218

OIPE IAP43 MAR 09 2006 PATENT & TRADEMARK OFFICE

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: March 6, 2006 Signature: (Mary Jane DiPalma)

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Wang Yi

Application No.: 11/127438

Confirmation No.: 5259

Filed: May 11, 2005

Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: Vandervegt, Francois P.

INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

Applicant has not submitted copies of each cited U.S. patent and U.S. patent application as required by 37 CFR 1.98(a)(2)(i), amended October 2004, as the U.S. Patent and Trademark Office has waived this requirement for all U.S. patent applications. Applicant submits herewith copies of foreign and non-patents in accordance with 37 CFR 1.98(a)(2). Also enclosed is a copy of a Search Report from a corresponding European application.

Those documents which are marked with a double asterisk (**) next to the Cite No. in the attached form PTO/SB/08 are not supplied because they were previously cited by or

Samsung Bioepis Exhibit No. 1002
Page 8219

submitted to the Office in a prior application number 10/655861, filed September 5, 2003 and relied upon in this application for an earlier filing date under 35 U.S.C. 120.

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: March 6, 2006

Respectfully submitted,

By _____

Xuqiong Wu, Ph.D.
Registration No.: 55,745
FISH & NEAVE IP GROUP
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

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Page 8220

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete If Known	
Application Number	11/127438
Filing Date	May 11, 2005
First Named Inventor	Wang Yi
Art Unit	1644
Examiner Name	Vandervegt, Francois P.
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA**	US-6,316,502	11-13-2001	Lai et al.	
	AB**	US-6,355,245	03-12-2002	Evans et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
	BA**	EP-0649468-B1	03-15-2000	AdProTech plc		

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Abe et al., "Contribution of Anaphylatoxin C5a to Late Airway Responses after Repeated Exposure of Antigen to Allergic Rats," The Journal of Immunology 167(8):4651-4660 (2001)	
	CB	Krug et al., "Complement Factors C3a and C5a are Increased in Bronchoalveolar Lavage Fluid after Segmental Allergen Provocation in Subjects with Asthma," Am. J. Respir. Crit. Care Med. 164:1841-1843 (2001)	
	CC**	Lukacs et al., "Complement-dependent Immune Complex-induced Bronchial Inflammation and Hyperreactivity," Am. J. Physiol. Lunch Cell Mol. Physiol. 280:L512-L518 (2001)	
	CD	Peng et al., "Role of C5 in the Development of Airway Inflammation, Airway Hyperresponsiveness, and Ongoing Airway Response," The Journal of Clinical Investigation 115(6):1590-1600 (2005)	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature		Date Considered	

Samsung Bioepis Exhibit No. 1002
Page 8221

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: September 22, 2006 Signature: (Mary Jane DiPalma)

IFW

Docket No.: ALXN-P02-102
(PATENT)

OIPE IAP80 SEP 25 2006 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: Vandervegt, Francois P.

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

Applicant submits herewith copies of non-patent literature in accordance with 37 CFR 1.98(a)(2).

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Samsung Bioepis Exhibit No. 1002
Page 8222

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: September 22, 2006

Respectfully submitted,

By _____

Xuqiong Wu, Ph.D.
Registration No.: 55,745
FISH & NEAVE IP GROUP, ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 3

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	Vandervegt, Francois P.
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CE	Abrahamsen et al., "Differential Mediator Release from Basophils of Allergic and Non-allergic Asthmatic Patients after Stimulation with Anti-IgE and C5a," Clinical and Experimental Allergy 31:368-378 (2001)	
	CF	Akatsu et al., "Distribution of Rat C5a Anaphylatoxin Receptor," Microbiol. Immunol. 46(12):863-874 (2002)	
	CG	Bjornson et al., "Complement is Activated in the Upper Respiratory Tract During Influenza Virus Infection," Am. Rev. Respir. Dis. 143:1062-1066 (1991)	
	CH	Blease et al., "Chemokines and Their Role in Airway Hyper-reactivity," Respir. Res. 1:54-61 (2000)	
	CI	Boulet et al., "Airway Hyperresponsiveness, Inflammation, and Subepithelial Collagen Deposition in Recently Diagnosed vs. Long-standing Mild Asthma," American Journal of Respiratory and Critical Care Medicine 162:1308-1313 (2000)	
	CJ	Chakir et al., "Airway Remodeling-associated Mediators in Moderate to Severe Asthma: Effect of Steroids on TGF-β, IL-11, IL-17, and Type I and Type III Collagen Expression," J. Allergy Clin. Immunol. 111:1293-1298 (2003)	
	CK	Chenoweth et al., "Demonstration of Specific C5a Receptor on Intact Human Polymorphonuclear Leukocytes," Proc. Natl. Acad. Sci. USA 75(8):3943-3947 (1978)	
	CL	Chung, "Cytokines in Chronic Obstructive Pulmonary Disease," Eur. Respir. J. 18(34):50s-59s (2001)	
	CM	Cieslewicz et al., "The Late, but not Early, Asthmatic Response is Dependent on IL-5 and Correlates with Eosinophil Infiltration," J. Clin. Invest. 104:301-308 (1999)	
	CN	Collard et al., "Complement Activation Following Oxidative Stress," Molecular Immunology 36:941-948 (1999)	
	CO	Czermak et al., "Complement, Cytokines, and Adhesion Molecule Expression in Inflammatory Reactions," Proceedings of the Association of American Physicians 110(5):306-312 (1998)	
	CP	Desai et al., "Demonstration of C5 Cleaving Activity in Bronchoalveolar Fluids and Cells: A Mechanism of Acute and Chronic Alveolitis," Journal of Experimental Pathology 1(3):201-216 (1984)	

Examiner Signature		Date Considered	

Samsung Bioepis Exhibit No. 1002
Page 8224

PTO/SB/08a/b (07-06)
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Substitute for form 1449A/B/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/127,438
				Filing Date	May 11, 2005
				First Named Inventor	Yi Wang
(Use as many sheets as necessary)				Art Unit	1644
				Examiner Name	Vandervegt, Francois P.
Sheet	2	of	3	Attorney Docket Number	ALXN-P02-102

	CQ	Drouin et al, "Expression of the Complement Anaphylatoxin C3a and C5a Receptors on Bronchial Epithelial and Smooth Muscle Cells in Models of Sepsis and Asthma," The Journal of Immunology 166:2025-2032 (2001)	
	CR	Frank, "Complement: A Brief Review," J. Allergy Clin. Immunol. 84(4,1):411-420 (1989)	
	CS	Gerard et al., "Complement in Allergy and Asthma," Current Opinion in Immunology 14:705-708 (2002)	
	CT	Glovsky et al., "Is Complement Activation a Factor in Bronchial Asthma?," Int. Arch. Allergy Immunol. 118:330-332 (1999)	
	CU	Gonczi et al., "The Severity of Clinical Symptoms in Ragweed-allergic Patients is Related to the Extent of Ragweed-induced Complement Activation in their Sera," Allergy 51:1110-1114 (1997)	
	CV	Hawlisch et al., "The Anaphylatoxins Bridge Innate and Adaptive Immune Responses in Allergic Asthma," Molecular Immunology 41:123-131 (2004)	
	CW	Hogaboam et al., "Mannose-binding Lectin Deficiency Alters the Development of Fungal Asthma: Effects on Airway Response, Inflammation, and Cytokine Profile," Journal of Leukocyte Biology 75:805-814 (2004)	
	CX	Holgate et al., "The Bronchial Epithelium as a Key Regulator of Airway Inflammation and Remodelling in Asthma," Clinical and Experimental Allergy 29:90-95 (1999)	
	CY	Höpken et al., "TheC5a Chemoattractant Receptor Mediates Mucosal Defence to Infection," Nature 383:86-89 (1996)	
	CZ	Irvin et al., "Airways Hyperreactivity and Inflammation Produced by Aerosolization of Human C5A des arg$^{1-3}$," Am. Rev. Respir. Dis. 134:777-783 (1986)	
	CA1	Jagels et al., "C3a and C5a Enhance Granulocyte Adhesion to Endothelial and Epithelial Cell Monolayers: Epithelial and Endothelial Priming is Required for C3a-induced Eosinophil Adhesion," Immunopharmacology 46:209-222 (2000)	
	CB1	Karp et al., "Identification of Complement Factor 5 as a Susceptibility Locus for Experimental Allergic Asthma," Nature Immunology 1(3):221-226 (2000)	
	CC1	Kodani et al., "Intratracheal Administration of Anaphylatoxin C5a Potentiates Antigen-induced Pulmonary Reactions Through the Prolonged Production of Cysteinyl-leukotrienes," Immunopharmacology 49:263-274 (2000)	
	CD1	Larsen et al., "A Differential Effect of C5a and C5a des Arg in the Induction of Pulmonary Inflammation," Am. J. Pathol. 100:179-192 (1980)	
	CE1	Maruo et al., "Generation of Anaphylatoxins Through Proteolytic Processing of C3 and C5 by House dust Mite Protease," J. Allergy Clin. Immunol. 100:253-260 (1997)	
	CF1	Mattos et al., "Matrix Metalloproteinase-9 Expression in Asthma," Chest 122:1543-1552 (2002)	
	CG1	Nagata et al., "Activation of Human Serum Complement with Allergens," J. Allergy Clin. Immunol.80:24-32 (1987)	
	CH1	Nagy et al., "The Development of Asthma in Children Infected with *Chlamydia pneumoniae* is Dependent on the Modifying Effect of Mannose-binding Lectin," J. Allergy Clin. Immunol. 112(4):729-734 (2003)	
	CI1	O'Byrne et al., "Reassessing the Th2 Cytokine Basis of Asthma," Trends in Pharmacological Sciences 25(5):244-248 (2004)	
	CJ1	Robbins et al., "Complement Activation by Cigarette Smoke," L254-L259 (1990)	
	CK1	Taube et al., "Inhibition of Complement Activation Decreases Airway Inflammation and Hyperresponsiveness," Am. J. Respir. Crit. Car Med. 168:1333-1341 (2003)	
	CL1	Teran et al., "Identification of Neutrophil Chemotactic Factors in Bronchoalveolar Lavage Fluid of Asthmatic Patients," Clinical and Experimental Allergy 27:396-405 (1997)	
	CM1	Varsano et al., "Generation of Complement C3 and Expression of Cell Membrane Complement Inhibitory Proteins by Human Bronchial Epithelium Cell Line," Thorax 55:364-369 (2000)	

Examiner Signature		Date Considered	

Samsung Bioepis Exhibit No. 1002
Page 8225

PTO/SB/08a/b (07-06)
Approved for use through 09/30/2006. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449A/B/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/127,438
				Filing Date	May 11, 2005
				First Named Inventor	Yi Wang
				Art Unit	1644
(Use as many sheets as necessary)				Examiner Name	Vandervegt, Francois P.
Sheet	3	of	3	Attorney Docket Number	ALXN-P02-102

	CN1	Wang et al., "Amelioration of Lupus-like Autoimmune Disease in NZB/WF$_1$ Mice after Treatment with a Blocking Monoclonal Antibody Specific for Complement Component C5," Proc. Natl. Acad. Sci. USA 93:8563-8568 (1996)	
	CO1	Wang et al., "Anti-C5 Monoclonal Antibody Therapy Prevents Collagen-induced Arthritis and Ameliorates Established Disease," Proc. Natl. Acad. Sci. USA 92:8955-8959 (1995)	
	CP1	Wills-Karp et al., "Interleukin-13: Central Mediator of Allergic Asthma," Science 282:2258-2261 (1998)	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature		Date Considered	

Samsung Bioepis Exhibit No. 1002
Page 8226

IFW

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 4-13-07 Signature: Maura A. Gallagher
~~Elaine Leahy~~
(Maura A. Gallagher)

OIPE APR 19 2007 PATENT & TRADEMARK OFFICE

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438

Confirmation No.: 5259

Filed: May 11, 2005

Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: F.P. Vandervegt

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

In accordance with 37 CFR 1.98(a)(2)(ii), Applicant has not submitted copies of U.S. patents and U.S. patent applications. Applicant submits herewith copies of foreign patents and non-patent literature in accordance with 37 CFR 1.98(a)(2).

10489597_1

Samsung Bioepis Exhibit No. 1002
Page 8227

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: 4/13/07

Respectfully submitted,

By Anita Varma

Anita Varma, Esq.
Registration No.: 43,221
FISH & NEAVE IP GROUP, ROPES & GRAY LLP
One International Place
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(617) 951-7000
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Attorneys/Agents For Applicant

Samsung Bioepis Exhibit No. 1002
Page 8228

OIPE IAP39 APR 19 2007 PATENT & TRADEMARK OFFICE

PTO/SB/08A/B (09-06)
Approved for use through 03/31/2007. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F.P. Vandervegt
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AC	US-4,228,795	10/21/1980	Babington	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CQ1	Fitch et al., "Pharmacology and Biological Efficacy of a Recombinant, Humanized, Single-Chain Antibody C5 Complement Inhibitor in Patients Undergoing Coronary Artery Bypass Graft Surgery with Cardiopulmonary Bypass," *Circulation* 100:2499-2506 (1999).	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature		Date Considered	

10489609_1

Samsung Bioepis Exhibit No. 1002
Page 8229

UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
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Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/127,438	05/11/2005	Yi Wang	ALXN-P02-102	5259

28120 7590 10/05/2007

ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

EXAMINER	
VANDERVEGT, FRANCOIS P	

ART UNIT	PAPER NUMBER
1644	

MAIL DATE	DELIVERY MODE
10/05/2007	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/127,438	WANG, YI	
	Examiner	Art Unit	
	F. Pierre VanderVegt	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-30* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *4-7* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____.
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Election/Restrictions

1. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claim 4, 6 and 7, drawn to a method for preventing or treating a pulmonary condition using an antibody to complement component C5 or a fragment thereof, classified in class 424, subclass 133.1.

II. Claim 5, drawn to a method for preventing or treating a pulmonary condition using an antibody to C5 convertase, classified in class 424, subclass 143.1.

Claims 1-3 and 8-30 link inventions I-II. The restriction requirement between the linked inventions is subject to the nonallowance of the linking claims, claims 1-3 and 8-30. Upon the allowance of the linking claim(s), the restriction requirement as to the linked inventions shall be withdrawn and any claim(s) depending from or otherwise including all the limitations of the allowable linking claim(s) will be entitled to examination in the instant application. Applicants are advised that if any such claim(s) depending from or including all the limitations of the allowable linking claim(s) is/are presented in a continuation or divisional application, the claims of the continuation or divisional application may be subject to provisional statutory.

The inventions are distinct, each from the other because of the following reasons:

2. Inventions I and II are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different designs, modes of operation, and effects (MPEP § 802.01 and § 806.06). In the instant case, the different inventions are drawn to the use of antibodies to different target proteins. Group I is drawn to antibodies that recognize a component of the complement cascade directly, while Group II is drawn to antibodies that react with an enzyme that modulates the cascade.

3. Because these inventions are independent or distinct for the reasons given above and there would be a serious burden on the examiner if restriction is not required because the inventions have acquired a

Samsung Bioepis Exhibit No. 1002
Page 8232

separate status in the art due to their recognized divergent subject matter, restriction for examination purposes as indicated is proper.

4. This application contains claims directed to the following patentably distinct species:

In Group I:

Antibodies to C5a.

Antibodies to the C5b-9 complex or a subunit thereof.

The species are independent or distinct because the target proteins are different and the effects of the target molecule in an immune response is different.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 1-4 and 8-30 are generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

5. Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species or invention to be examined even though the requirement be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention or species may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse.

Should applicant traverse on the ground that the inventions or species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions or species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the

Samsung Bioepis Exhibit No. 1002
Page 8233

examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C.103(a) of the other invention.

6. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

7. Any inquiry concerning this communication or earlier communications from the examiner should be directed to F. Pierre VanderVegt whose telephone number is (571) 272-0852. The examiner can normally be reached on M-Th 6:30-4:00 and Alternate Fridays 6:30-3:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Christina Chan can be reached on (571) 272-0841. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

F. Pierre VanderVegt, Ph.D. /PV/
Patent Examiner
October 1, 2007

David A. Saunders

DAVID A. SAUNDERS
PRIMARY EXAMINER

Samsung Bioepis Exhibit No. 1002
Page 8234

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 11/5/07 Signature: Ginny Blundell

OIPE
NOV 07 2007
PATENT & TRADEMARK OFFICE

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Vandervegt, Francois P.

RESPONSE TO RESTRICTION REQUIREMENT

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In response to the restriction requirement set forth in the Office Action mailed October 5, 2007, applicant hereby provisionally elects Group I (claims 4, 6 and 7) for continued examination.

The Examiner has also required an election of species between C5a and the C5b-9 complex or a subunit thereof. Applicant notes that in addition to these species, claim 4 lists C5 which is a distinct molecule from C5a and C5b-9. Applicant hereby elects C5 as the species for search purposes only.

Claims 1-4 and 7-30 read on the elected species.

Samsung Bioepis Exhibit No. 1002
Page 8235

Applicant believes no fee is due with this response. However, if a fee is due, please charge our Deposit Account No. 18-1945, under Order No. ALXN-P02-102 from which the undersigned is authorized to draw.

Dated: November 5, 2007

Respectfully submitted,

By _____

Anita Varma, Esq.
Registration No.: 43,221
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

OIPE
FEB 25 2008
PATENT & TRADEMARK OFFICE

PTO/SB/21 (01-08)
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U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F. P. Vandervegt
Attorney Docket Number	ALXN-P02-102

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [x] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

Form PTO SB/08
References BB-BC and CR1-CS1
Return Postcard

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	ROPES & GRAY LLP		
Signature			
Printed name	Ryan Murphey, Ph.D.		
Date	February 21, 2008	Reg. No.	61,156

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: February 21, 2007 Signature:  (Andrea Silverman)

3543561_1.DOC

Samsung Bioepis Exhibit No. 1002
Page 8237

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: February 21, 2007 Signature: Andrea Silverman (Andrea Silverman)

OIPE FEB 25 2008 PATENT & TRADEMARK OFFICE

Docket No.: ALXN-P02-102 (PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES Examiner: F. P. Vandervegt

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

Applicant submits herewith copies of foreign patents and non-patent literature in accordance with 37 CFR 1.98(a)(2).

Further, Applicants bring the following co-pending patent application and the Office Actions issued in the co-pending application to the Examiner's attention:

Application No.	Title	Filed	Atty. Dkt. No.
10/655,861	METHOD OF TREATMENT OF ASTHMA USING ANTIBODIES TO COMPLEMENT COMPONENT C5	September 5, 2003	ALXN-P01-102

1. Office Action dated March 13, 2007
2. Office Action dated September 10, 2007

Applicants will be happy to provide copies of any of the applications or office actions cited upon request by the Examiner.

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102. A duplicate copy of this paper is enclosed.

Dated: February 21, 2008

Respectfully submitted,

By _____

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(54) Title: METHOD OF TREATMENT OF ASTHMA USING ANTIBODIES TO COMPLEMENT COMPONENT C5

(57) Abstract: A method of treatment of asthma with a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components such as, for example, a complement-inhibiting antibody. The compound can be administered prophylactically to prevent asthma attacks in known asthmatic individuals or as a therapeutic regimen during asthma attacks.

Samsung Bioepis Exhibit No. 1002
Page 8241

METHOD OF TREATMENT OF ASTHMA USING ANTIBODIES TO COMPLEMENT COMPONENT C5

BACKGROUND

TECHNICAL FIELD

This disclosure relates to a method of treating asthma using a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components such as, for example, a complement-inhibiting antibody.

DESCRIPTION OF THE RELATED ART

Asthma, bronchitis and emphysema are known collectively as Chronic Obstructive Pulmonary Diseases. These diseases are characterized as generalized airways obstruction, especially of small airways, associated with varying degrees of symptoms of chronic bronchitis, asthma, and emphysema. These diseases may often coexist in an individual, and it may be difficult to determine the primary cause of an airway obstruction. Airway obstruction is defined as an increased resistance to airflow during forced expiration. Obstruction of large airways may also occur in these diseases, particularly in asthma.

Asthma is a reversible obstructive pulmonary disorder caused by an airway hyper-responsiveness to specific and/ or non-specific stimuli. Asthmatic airway obstruction typically results from bronchospasms. Asthma may be

Samsung Bioepis Exhibit No. 1002
Page 8242

triggered by a variety of causes such as allergic reactions, a secondary response to infections, industrial or occupational exposures, ingestion of certain chemicals or drugs, exercise, and vasculitis. Much of asthma's pathology can be attributed to mast cell degranulation. Mast cells will degranulate in response to various conditions such as, for example, classical IgE-antigen stimulation. It is believed that when the asthmatic, human or animal, inhales an allergenic substance, sensitized IgE antibodies trigger mast cell degranulation in the lung interstitium. The mast cell degranulation releases histamine, bradykinin, and slow-reacting substance of anaphylaxis (SRS-A) which includes the leukotrienes C, D and E, prostaglandins including PGF_2, $PGF_{2\alpha}$, and PGD_2, and thromboxane A_2. The histamine then attaches to receptor sites in the larger bronchi, causing irritation, inflammation, and edema. The SRS-A attaches to receptor sites in the smaller bronchi, causing edema and attracting prostaglandins, which enhance the effects of histamine in the lungs. Histamine, in combination with prostaglandins, also stimulates excessive mucous secretion, narrowing the bronchial lumen further. When an asthmatic individual inhales, the narrowed bronchial lumen still expands slightly, allowing air to reach the alveoli. However, upon exertion to exhale, the increased thoracic pressure closes the bronchial lumen completely. Therefore, air can enter the lungs, but may not exit during an asthma attack. The ventilation in the alveoli is then inhibited by mucous collecting in the lung bases. In an effort to compensate for lowered alveolar ventilation, blood is shunted to other alveoli. Hypoxia, and in extreme cases, respiratory acidosis may result without medical intervention. In many cases, there are two phases to an allergic asthma attack,

2

Samsung Bioepis Exhibit No. 1002
Page 8243

an early phase and a late phase which follows 4-6 hours after bronchial stimulation. The early phase includes the immediate inflammatory response including the reactions caused by the release of cellular mediators from mast cells (i.e., histamine). Late phase reactions develop over a period of hours and are characterized histologically by an early influx of polymorphonuclear leukocytes and fibrin deposition, later followed by infiltration of eosinophils. Increased levels of eosinophil-derived inflammatory mediators in plasma and BAL, including eosinophilic cationic protein and major basic protein, have been observed during the late phase reaction. Upregulation of TH2 -type cytokines (IL4, IL5 and IL 13) following allergen challenge has also been observed during the late phase. Thus, the cellular inflammatory response, in combination with released pro-inflammatory mediators (e.g., mmp9) and locally produced cytokines in the bronchial mucosa, play a central role in the late phase allergic inflammation and bronchoconstriction. Late phase reactions increase airway reactivity and lead to prolonged asthmatic exacerbations that may last from hours to days to months in some subjects. One of the residual effects of asthma reactions is this hyperresponsiveness of the airways to nonspecific stimuli.

Currently, the treatments for asthma are not always adequate and many have serious side effects. The general goals of drug therapy for asthma are prevention of bronchospasm and control of airway hyperreactivity or hyperresponsiveness, an indication of airway inflammation. It is very difficult to eliminate or prevent exposure to all allergens that may trigger an asthma attack.

Samsung Bioepis Exhibit No. 1002
Page 8244

To prevent these attacks, most asthmatics are treated with various pharmacological agents, many of which have side effects.

In a study reported by Lukacs et al. (Lukacs at el, Am. J. Physiol Lung Cell Mol Physiol. 2001), anti-C5a antibodies were administered intratracheally togeher with anti-BSA antibody during the induction of immune complex mediated lung inflammation. Specifically, the Lukacs study uses a model of acute immune complex mediated tissue lung inflammation similar to reverse passive Arthus reaction in skin, with a brief AHR to intravenous challenges of methacholine. Animals did not develop chronic airway inflammation featured by eosinophilia; nor did they experience previous severe asthmatic attack after exposed to allergens as demonstrated in the current invention. The key feature of Lukacs study was the formation of immune complexes of BSA and anti-BSA locally along the airway, which activate the complement cascade and produced significantly amount of terminal complement components after intratracheally injected anti-BSA antibody into animals. The subsequent development of AHR to methacholine lasting up to four hours during the acute phase BSA-anti-BSA induced lung inflammation, which is in significantly contrast to the severe and long lasting AHR seen patients with asthma. It is reasonable to assume based on the Lukacs study that anti-C5a antibody neutralizes C5a produced locally in the airway and therefor prevents the development of harmful downstream events mediated by C5a such as recruiting and activating of inflammatory cells, stimulating the release of mediators and vascular leaky syndromes. The combination effects of blocking C5a mediated recruitment of inflammatory cells,

Samsung Bioepis Exhibit No. 1002
Page 8245

release of mediators such as histamine, and the development of edema of airways that leads to the reduction of airway resistance and prevention of the development of AHR when animals were challenged with IV methacholine. This study may suggest the importance of C5a in development of AHR of immune complexes mediated lung inflammation. However, this study provides no basis to predict by if there is any direct and immediate effect of bronchial dilation by C5 inhibitors during an on-going asthmatic attack or late phase airway responses to allergens. Nor does this study involve the treatment of subjects that have established airway inflammation or subjects that have experienced previous asthmatic symptoms.

SUMMARY

The present disclosure relates to a treatment for asthma using a compound which binds to or otherwise blocks the generation and/or activity of one or more complement components or blocks the engagement of complement component receptors, such as, for example, C5a receptors. The treatment therapy includes the administration of a compound that inhibits the production and/or activity of at least one complement component. Suitable compounds include, for example, antibodies which bind to or otherwise block the generation and/or activity of one or more complement components, such as, for example, an antibody specific to complement component C5.

The complement-inhibiting compound can be administered prophylactically in known asthmatic individuals (such as those having established airway inflammation or a subject that has experienced previous asthmatic

symptoms) to prevent, or help prevent asthma attacks. This prophylactic therapy can be administered via intravenous, aerosol, subcutaneous or intramuscular routes.

The complement-inhibiting compound can be administered as a therapeutic regimen to an individual experiencing an asthma attack. The regimen can be administered via intravenous, aerosol, subcutaneous or intramuscular routes.

A combination therapy may also be used that includes a complement-inhibiting compound in combination with a regimen of known asthma therapy, such as, for example, steroids, anti-IgE antibodies, anti-IL-4 antibodies, anti-IL-5 antibodies, β2 receptor agonists, leukotriene inhibitors, 5 Lipoxygenase inhibitors, b2 adreno receptor agonists, PDE inhibitors, IL 5 antagonists, CD23 antagonists, IL 13 antagonists, cytokine release inhibitors, histamine H1 receptor antagonists, anti-histamines and histamine release inhibitors. Suitable compounds of each class listed above as well as other asthma treatments are listed in Asthma Therapeutic: New Treatment Options and Emerging Drug Discovery Tasrgerts, Barnes, April 2003, Lead Discovery, http://www.leaddiscovery.co.uk/target-discovery/abstracts/dossier-asthma.html

In another aspect, a method of reducing inflammation in the lungs of asthma patients is described herein. The methods include the step of administering to a subject having or susceptible to asthma a compound that reduces the release or production of inflammatory mediators (such as, for example, matrix metalloprotease 9 (mmp9 - also known as the 92-kDa type IV

Samsung Bioepis Exhibit No. 1002
Page 8247

collagenase/gelatinase or gelatinase B), TGFβ, eosinophil granules, and the like) in the airways of the subject. The compound can act at the cellular level to reduce the production or release of the inflammatory mediator, can interact with the inflammatory mediator in a manner that interfaces with its activity, (such as, for example by preventing the conversion of pro-mmp-9 to the 83 kDa active form or can interact with an active form) of the inflammatory mediator to prevent the inflammatory effects associated therewith.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 graphically shows the OVA-induced asthmatic reactions in normal BALB/c Mice.

Figure 2a shows the schedule and nature of antigen challenges and prophylactic treatment of normal BALB/c Mice.

Figure 2b shows a schedule of antigen challenge and prophylactic treatment in normal BALB/c Mice.

Figure 3 graphically summarizes the effects of the treatments shown in Figures 2a and 2b.

Figure 4a shows the schedule and nature of antigen challenges and IV or aerosol treatment of normal BALB/c Mice during an asthma attack.

Figure 4b depicts the protocol and results of induction of asthmatic attack in BALB/c Mice.

Figures 5a and 5b graphically summarize the effects of the aerosol treatments shown in Figure 4a.

Figures 6a and 6b graphically summarize the effects of the intravenous treatments shown in Figure 4a.

Figure 7 graphically depicts the systemic effects of various treatments on C5 activity for treatments shown in Figure 4a.

Figure 8 graphically shows the effect of various intravenous treatments on the total WBC count in BAL.

Figures 9 and 10 show that eosinophils were found to be the predominant inflammatory cells in BAL.

Figure 11 graphically shows the effect of various intravenous treatments on histamine and in BAL.

Figure 12 graphically shows the effect of various intravenous treatments on MMP-9 levels in BAL

Figure 13 graphically shows the effect of various intravenous treatments on TGFβ levels in BAL.

Figure 14 shows the schedule and nature of antigen challenges and cannulation and aerosol treatment of normal BALB/c Mice during an asthma attack.

Figure 15 shows lung resistance in asthmatic mice treated during an asthma attack with anti-C5, β2 receptor agonist or a combination thereof.

DETAILED DESCRIPTION

The present disclosure is directed to a method of treating asthma in mammals. Specifically, the methods of treating asthma described herein involve using compounds which bind to or otherwise block the generation and/or activity

of one or more complement components. The inhibition or blocking of the generation of complement components inhibits multiple factors involved in the broncho-constrictive responses in asthma. A specific class of such compounds which are particularly useful are antibodies specific to a human complement component, especially anti-C5 antibodies.

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions. A concise summary of the biologic activities associated with complement activation is provided, for example, in The Merck Manual, 16th Edition.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative

Samsung Bioepis Exhibit No. 1002
Page 8250

pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Additionally, the lectin pathway is typically initiated with binding of mannose-binding lectin (MBL) to high mannose substrates. These pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin. C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is generally considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone.

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways. This property of C3b is regulated by the serum protease Factor I, which acts on

Samsung Bioepis Exhibit No. 1002
Page 8251

C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75 µg/ml (0.4 µM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence (see, U.S. patent 6,355,245).

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment (amino acid residues +1 to 655 of the above sequence) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of the above sequence), with four amino acids (amino acid residues 656-659 of the above sequence) deleted between the two.

C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733 of the above sequence). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at, or immediately adjacent to, amino acid residue 733 of the above sequence. A compound that would bind at, or adjacent, to this cleavage site would have the potential to block

Samsung Bioepis Exhibit No. 1002
Page 8252

access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

Cleavage of C5 releases C5a, a potent anaphylatoxin and chemotactic factor, and leads to the formation of the lytic terminal complement complex, C5b-9. C5a and C5b-9 also have pleiotropic cell activating properties, by amplifying the release of downstream inflammatory factors, such as hydrolytic enzymes, reactive oxygen species arachidonic acid metabolites and various cytokines.

C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex--TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases

histamine from basophils and mast cells, and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

C5a receptors are found on the surfaces of bronchial and alveolar epithelial cells and bronchial smooth muscles cells. C5a receptors have also been found on eosinophils, mast cells, monocytes, neutrophils, and activated lymphocytes. Thus, compounds that block engagement of receptors of complement components are useful herein.

Any compounds which bind to or otherwise block the generation and/or activity of any of the human complement components, such as, for example, antibodies specific to a human complement component are useful herein. Some compounds include antibodies directed against complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, AND MASP-2, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. Also useful in the present methods are naturally occurring or soluble forms of complement inhibitory compounds such as CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, y bind protein, complestatin, and K76 COOH.

Samsung Bioepis Exhibit No. 1002
Page 8254

Particularly useful compounds for use herein are antibodies that reduce, directly or indirectly, the conversion of complement component C5 into complement components C5a and C5b. One class of useful antibodies are those having at least one antibody-antigen binding site and exhibiting specific binding to human complement component C5, wherein the specific binding is targeted to the alpha chain of human complement component C5. More particularly, a monoclonal antibody (mAb) may be used. Such an antibody 1) inhibits complement activation in a human body fluid; 2) inhibits the binding of purified human complement component C5 to either human complement component C3 or human complement component C4; and 3) does not specifically bind to the human complement activation product for C5a. Particularly useful complement inhibitors are compounds which reduce the generation of C5a and/or C5b-9 by greater than about 30%. Anti-C5 antibodies that have the desirable ability to block the generation of C5a have been known in the art since at least 1982 (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, supra; Mollnes et al. Scand. J. Immunol. 1988, 28:307-312); C5a (see for example, Ames et al. J. Immunol. 1994, 152:4572-4581, U.S. Pat. No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201-209). Particularly useful anti-C5

Samsung Bioepis Exhibit No. 1002
Page 8255

antibodies are h5G1.1, h5G1.1-scFv and functional fragments of h5G1.1. Methods for the preparation of h5G1.1, h5G1.1-scFv and functional fragments of h5G1.1 are described in U.S. Patent No. 6,355,245 and "Inhibition of Complement Activity by Humanized Anti-C5 Antibody and Single Chain Fv", Thomas et al., *Molecular Immunology*, Vol. 33, No. 17/18, pages 1389-1401, 1996, the disclosures of which are incorporated herein in their entirety by this reference.

Functionally, a suitable antibody inhibits the cleavage of C5, which blocks the generation of potent proinflammatory molecules C5a and C5b-9 (terminal complement complex). Preferably, the antibody does not prevent the formation of C3b, which subserves critical immunoprotective functions of opsonization and immune complex clearance.

While preventing the generation of these proinfammatory terminal complement components, antibody-mediated inhibition of the complement cascade at C5 preserves the ability to generate C3b, which is critical for opsonization of many pathogenic microorganisms, as well as for immune complex solubilization and clearance. Retaining the capacity to generate C3b appears to be particularly important as a therapeutic factor in complement inhibition for inflammatory diseases, where increased susceptibility to infection and impaired clearance of immune complexes are preexisting clinical features of the disease process.

Samsung Bioepis Exhibit No. 1002
Page 8256

The anti-human C5 antibody is preferably a monoclonal antibody, although polyclonal antibodies produced and screened by conventional techniques can also be used if desired.

The preferred anti-C5 antibodies used to treat asthma in accordance with this disclosure bind to C5 or fragments thereof, e.g., C5a or C5b. Preferably, the anti-C5 antibodies are immunoreactive against epitopes on the alpha chain of purified human complement component C5 and are capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. This capability can be measured using the techniques described in Wurzner, et al., Complement Inflamm 8:328-340, 1991.

In a particularly useful embodiment, the anti-C5 antibodies are not immunoreactive against epitopes on the beta chain, but rather are immunoreactive against epitopes within the alpha chain of purified human complement component C5. In this embodiment, the antibodies are also capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. Within the alpha chain, the most preferred antibodies bind to an amino-terminal region, however, they do not bind to free C5a.

Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Pat. No. 5,135,916. Antibodies are prepared using purified components of the complement membrane attack complex as immunogens according to known methods. In accordance with this disclosure, complement component C5 or C5b

is preferably used as the immunogen. In accordance with particularly preferred useful embodiments, the immunogen is the alpha chain of C5.

Particularly useful antibodies share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete for binding to portions of C5--the C5 alpha chain; and

(2) they specifically bind to the C5 alpha chain. Such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth. 1993, 160:191-198).

(3) they block the binding of C5 to either C3 or C4 (which are components of C5 convertase).

In another aspect, a method of reducing inflammation in the lungs of asthma patients is provided. The methods include the step of administering to a subject having or susceptible to asthma a compound that reduces the production or release of inflammatory mediators in the airway of the subject. Non-limiting examples of inflammatory mediators that can be reduced in accordance with this disclosure include TGFβ, eosinophil granule proteins, and matrix metalloprotease 9 (mmp9 - also known as the 92-kDa type IV collagenase/gelatinase or gelatinase B). The compounds can reduce inflammation by any variety of mechanisms. Where the inflammatory mediator is MMP-9, for example, the compound can act at the cellular level to reduce the production or release of pro-mmp-9, can interact with pro-mmp-9 in a manner that prevents conversion of pro-

Samsung Bioepis Exhibit No. 1002
Page 8258

mmp-9 to the 83 kDa active form or can interact with the active form of mmp9 to prevent the inflammatory effects associated with the enzyme (such as, for example, the generation of TGF-beta). Suitable compounds include, but are not limited to, the compounds described above which bind to or otherwise block the generation and/or release an/or activity of one or more complement components or block engagement of the receptors of complement components.

MMP-9 activity can be detected in accordance with a variety of art-recognized procedures. For example, quantitative zymographic methods provide a relatively refined assessment of the activity of this enzyme. This method allows for the detection of MMP-9 activity based upon the ability of the enzyme to hydrolyze denatured collagen, i.e., gelatin, which is a natural substrate for MMP-9. The gelatin is incorporated into a gel such as polyacrylamide. See Hibbs et al., J. Biol. Chem. 260:2493-2500 (1985) and Moll et al., Cancer Res. 50:6162-70 (1990). The assay may be standardized using a purified MMP-9 preparation that is analyzed in parallel with the test sample. Purified MMP-9 can be prepared by methods known in the art. See, for example, Okada et al., supra., and Morodomi et al., Biochem J. 285:603:11 (1992). The extent of hydrolysis of the gelatin is directly related to the activity of MMP-9 in the sample, and the active MMP-9 forms can be identified by their characteristic molecular weights. In the gelatin zymography, the proMMP-9 species can be detected because of the catalytic activation that occurs during electrophoresis and subsequent incubation. However, the MMP-9 forms present prior to the onset of labor are incapable of undergoing this kind of activation, i.e., they are latent.

Samsung Bioepis Exhibit No. 1002
Page 8259

MMP-9 activity can also be detected using standard immunological techniques, e.g., ELISA, immunofluorescence assays, or radioimmunoassays. In a preferred embodiment, MMP-9 activity is detected using ELISA, which entails the use of antibodies specific to MMP-9. See David et al, U.S. Pat. No. 4,376,110 (and references cited therein). Monoclonal antibodies specific to MMP-9 have been prepared using partially purified enzyme preparations. See, e.g., Moll et al., supra; Ramos-DeSimone et al., HYBRIDOMA 12(4):349-63 (1993) and Goldberg et al. Polyclonal antibodies specific to MMP-9 can also be prepared in accordance with standard procedures. In a preferred embodiment, polyclonal antibodies are prepared using non-conserved peptides conjugated to a macromolecular carrier. The choice of a specific non-conserved peptide such as the metal-binding domain, among the members of the MMPs is considered within a level of ordinary skill in the art. See Woessner, and Goldberg et al., supra. Enzymic assays that can detect MMP-9 in picogram or nanogram amounts are also disclosed in Manicourt et al., Anal. Biochem. 215(2):171-9 (1993).

MMP-9 activity can further be detected in a sample by western blot analysis, which requires electrophoretic separation of the test material in a gel, followed by transfer of the separated proteins to a nitrocellulose membrane and detection of the MMP-9 antigens with a specific antibody and reagent that reacts with the antigen-fixed antibody. See Towbin et al., Proc. Natl. Acad. Sci. USA 76(9):4350-4354 (1979).

Samsung Bioepis Exhibit No. 1002
Page 8260

Suitable assays for detection of mmp9 are commercially available from a variety of sources such as, for example Boehringer Manheim Biochemicals (Manheim, Germany) and R&D Systems, (Minneapolis, MN).

The compound that inhibits the production and/or activity of at least one complement component can be administered in a variety of unit dosage forms. The dose will vary according to the particular compound employed. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

Administration of the compound that inhibits the production and/or activity of at least one complement component will generally be in an aerosol form with a suitable pharmaceutical carrier, via intravenous infusion by injection, or subcutaneous injection. Other routes of administration may be used if desired. Aerosol administration is preferred since it avoids the systemic effects of the complement-inhibiting compound, while providing the desired asthma-treating effects to be achieved in accordance with this disclosure.

Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, Pa., 17th ed.

Samsung Bioepis Exhibit No. 1002
Page 8261

(1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

EXAMPLE 1-The Use Of Anti-C5 Antibodies As A Prophylactic For C5 Inhibition To Treat Asthma.

Asthma was induced in normal BALB/c mice by exposing them to Ovalbumin antigen ("OVA") and Alum, according to the dosages and schedule shown in Figure 4b. These exposures solicited the standard asthmatic response during an attack. The exposed mice showed both early phase and late phase reactions. As seen in Figure 1, the early phase reaction was specific airway resistance within 15 minutes after exposure. A more severe late phase reaction of specific airway resistance occurred approximately 5 hours after exposure. Specific airway resistance was measured by means of double chamber plethysmograph fitted with pneumotachographs, which is commercially available from the Buxo Corporation.

To demonstrate the prophylactic benefits of a complement component inhibiting compound, groups of mice were given one of three different treatments in accordance with the schedule of dosages shown in Figures 2a and 2b. The positive control group was treated with a control antibody (hybridoma 135.8) at a dose of 40 mg/kg. A second group of mice was treated with the anti-C5 antibody, BB5.1, at a dose of 40mg/kg. A third group of mice was treated with

Samsung Bioepis Exhibit No. 1002
Page 8262

Dexamethasone ("DEX"). A negative control group was initially exposed to PBS (phosphate buffer saline) and Alum, and was later given PBS as shown in the schedule of Figure 2a. This control group provided a baseline air resistance measurement.

The BB5.1 antibody is made according to known methods. (See, Frei, Y., Lambris, J.D., Stockinger, B. Mol. Cell. Probes. 1: 141-149 (1987)). Both the BB5.1 antibody and the isotype match control 135.8 hybridoma antibody were grown as ascites in athymic mice and the antibodies were purified from ascites by protein A affinity chromatography followed by elution with ImmunoPure IgG elution buffer (Pierce) and dialysis against PBS buffered saline. (Wang et al. 1996).

It was found that the group prophylactically treated subcutaneously with Anti-C5 antibody responded as well as the challenged mice treated with steroids and the negative control, as shown in Figure 3. The Anti-C5 treatments had inhibited the production of complement component C5 in the cascade, and by inhibiting C5 production, the mice experienced significantly less airway constriction. The positive control group that was treated with a control antibody showed increased specific airway resistance caused by an asthmatic attack.

Samsung Bioepis Exhibit No. 1002
Page 8263

EXAMPLE 2 - The Use Of Anti-C5 Antibodies Via Intravenous Administration As A Therapeutic Method To Treat Asthma

To demonstrate the therapeutic benefits of administering a C5 complement component inhibitor intravenously during an asthma attack, groups of mice were given one of three different treatments in accordance with the schedule of dosages shown in Figure 4a. A positive control group was challenged with OVA and treated with a control antibody (135.8 hybridoma) administered intravenously, 15 minutes after provoking the initial asthmatic attack. A second group of mice were similarly challenged and intravenously treated with BB5.1, the anti-C5 antibody, 15 minutes after provoking the initial asthmatic attack. A third group of mice were similarly challenged and intravenously treated with DEX, 15 minutes after provoking the initial asthmatic attack. Sham operated mice provided baseline air resistance measurement which was provided by administering phosphorate buffer solution (PBS) and alum to a group of mice, and administering aerosol doses of PBS according to the schedule shown on Fig. 4a.

The results of this challenge (shown in Figs. 6a and 6b) found that the group of mice treated therapeutically with anti-C5 intravenously showed very little specific airway constriction and these mice responded to the treatments as well as the group treated with the steroid, DEX. The positive control group that was treated with a control antibody showed significantly increased specific airway resistance, as shown in Figure 6a. The anti-C5 antibody had inhibited the

Samsung Bioepis Exhibit No. 1002
Page 8264

inflammatory response of the complement components, and allowed greater air passage during the asthma attacks in the mice.

EXAMPLE 3 - The Use Of Anti-C5 Antibodies Via Aerosol Administration As A Therapeutic Method To Treat Asthma

To demonstrate the therapeutic benefits of administering a C5 complement component inhibitor via aerosol during an asthma attack, groups of mice were given one of three different treatments in accordance with the schedule of dosages shown in Figure 4a. A positive control group was challenged with OVA and treated with a control antibody (135.8 hybridoma) administered by aerosol, 15 minutes after provoking the initial asthmatic attack. A second group of mice were similarly challenged and treated via aerosol administration of BB5.1, an anti-C5 antibody, 15 minutes after provoking the initial asthmatic attack. A third group of mice were similarly challenged and treated by aerosol with DEX, 15 minutes after provoking the initial asthmatic attack. A baseline air resistance measurement was provided by administering phosphorate buffer solution (PBS) and alum to a group of mice, and administering aerosol doses of PBS according to the schedule shown on Fig. 4a.

It was found that therapeutic aerosol treatments of mice with anti-C5 antibody, BB5.1 during asthma attacks significantly lowered the specific airway resistance and these mice responded as well as, and more quickly than, the steroid treated mice. The results of the aerosol treatments are shown in Figures 5a and 5b. The positive control group that was treated with a control antibody

Samsung Bioepis Exhibit No. 1002
Page 8265

experienced specific airway resistance many times greater than the mice treated with anti-C5 antibody, as shown in Figure 5b.

Anti-C5 antibody, BB5.1 was also administered to the test animals through nebulization, which was found to be an effective method of administration. Results of serum tests after nebulization indicate that the BB5.1 was binding with the C5 site and, therefore, remained intact during delivery through nebulization.

The systemic effect of each treatment given in Examples 2 and 3 was measured using the techniques described in Wurzner, et al., Complement Inflamm 8:328-340, 1991 for hemolytic activity. The results of these tests are shown in Figure 7. As seen therein, the control antibody and steroid did not substantially reduce systemic C5 activity, irrespective of the method of administration. With the anti-C5 antibody BB5.1, however, the manner of administration directly affected systemic C5 activity. Specifically, although both aerosol and intravenous administration were effective at reducing the severity of an asthma attack, the aerosol administration did so without substantially reducing systemic C5 activity. As seen in Figure 7, intravenous administration of the anti-C5 antibody reduced systemic C5 activity by nearly 80%.

EXAMPLE 4 - The Use Of Anti-C5 Antibodies To Reduce the Presence of Inflammatory mediators

To demonstrate the therapeutic benefits of administering a C5 complement component inhibitor in reducing the presence of inflammatory mediators during an asthma attack, groups of mice were given one of three different treatments in accordance with the schedule of dosages shown in Figure

Samsung Bioepis Exhibit No. 1002
Page 8266

4a. A positive control group was challenged with OVA and treated with a control antibody (135.8 hybridoma) administered intravenously, 15 minutes after provoking the initial asthmatic attack. A second group of mice were similarly challenged and intravenously treated with BB5.1, the anti-C5 antibody, 15 minutes after provoking the initial asthmatic attack. A third group of mice were similarly challenged and intravenously treated with DEX, 15 minutes after provoking the initial asthmatic attack. Sham operated mice provided baseline air resistance measurement which was provided by administering phosphorate buffer solution (PBS) and alum to a group of mice, and administering aerosol doses of PBS according to the schedule shown on Fig. 4a. After 5 hours the mice were euthanized and the lungs were lavaged using conventional techniques. Generally, 1 cc of PBI saline was introduced into the lungs and recovered. This process was repeated three times. The fluid was recovered and centrifuged. The cells contained in the resulting pellet were inspected. The supernatant was tested for the presence of histamine, IL-5, IL-4, IL-13, Eosinophils granule proteins, TGFβ and/or mmp-9. The assays were all conducted using commercially available test kits. The results are shown in Figures 8 through 13. These BronchoAlveolarLavage (*BAL*) results show the presence or absence of proteins which were produced or released by lung structure cells or inflammatory cells, rather than the presence of such components in the lung tissue itself.

Samsung Bioepis Exhibit No. 1002
Page 8267

It was found that treatment of mice with anti-C5 antibody and DEX both reduced the total WBC count. (See Fig. 8). Eosinophils were found to be the predominant inflammatory cells in the BAL. (See Figs. 9A-D and 10.)

Figure 11 shows that the anti-C5 antibody had little effect on histamine level, a result similar to that obtained with the steroid DEX. However, the anti-C5 antibody significantly reduced the level of mmp-9 detected in BAL as shown in Figure 12, compared to treatment with Steroid. Thus, while both anti-C5 antibody and DEX resulted in a lower detectable amount of TGF-β (see Fig. 13), only the anti-C5 antibody did so through a mechanism that involved a reduction in production or release of mmp-9.

EXAMPLE 5 - The Use Of Anti-C5 Antibodies As Bronchial Dilator

To demonstrate the direct and immediate bronchial dilation effect of administering a C5 complement component inhibitor (alone or in combination with a β2 adreno receptor agonist), during an asthma attack, groups of mice were challenged with antigen and given one of four different treatments in accordance with the schedule of dosages shown in Figures 14 and 15. A positive control group was challenged with OVA, cannulated and treated with a control antibody (mouse IgG1) administered via aerosol after provoking a second asthmatic attack. A second group of mice were similarly challenged and treated with BB5.1, an anti-C5 antibody after provoking a second asthmatic attack. A third group of mice were similarly challenged and treated with salbutamol (a β2 adreno receptor agonist commercially available from Sigma) after provoking a second asthmatic attack. A fourth group of mice were similarly challenged and treated

Samsung Bioepis Exhibit No. 1002
Page 8268

with a combination of anti-C5 antibody and salbutamol after provoking a second asthmatic attack. Sham operated mice provided baseline air resistance measurement which was provided by administering phosphorate buffer solution (PBS) and alum to a group of mice, and administering aerosol doses of PBS according to the schedule shown on Figs. 14 and 15.

Airway responsiveness was then assessed as a change in airway function by an invasive method wherein changes in lung resistance were measured by using Buxco Biosystem software and whole body plethysmograph. Mice were anesthetized with Avertin (160mg/kg) by i.p. injection and ventilated by a Harvard Apparatus Inspira ventilator. After tracheal cannula, pancuronium (0.3mg/kg) was injected intraperitoneally to induce paralysis and inhibit spontaneous breathing. The mice are kept breathing by a ventilator, which is set at a tidal volume and respiratory rate by program of body weight. The measurements of RL to specific antigen were performed at 5 hours after 5% OVA provocation. The results, which are reported in Figure 15 show that the treatment with anti-C5 antibody had a significant bronchial dilation effect in 3 of 4 mice and that a synergistic effect is observed from the treatment with anti-C5 antibody in combination with a β2 adreno receptor agonist.

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention.

CLAIMS

What is claimed is:

1. A method of treating asthma in a subject comprising administering an anti-C5 antibody to a subject susceptible to or having asthma.
2. A method of preventing asthma attacks comprising prophylactically administering an anti-C5 antibody to a subject having established airway inflammation or a subject that has experienced previous asthmatic symptoms.
3. A method of reducing the severity of an asthma attack comprising administering an anti-C5 antibody to a subject having an asthma attack.
4. A method of reducing airway obstruction in a subject comprising administering an anti-C5 antibody to the subject.
5. A method of increasing air flow in a subject comprising administering an anti-C5 antibody to the subject.
6. A method of reducing bronchial spasms in a subject comprising administering an anti-C5 antibody to the subject.
7. A method of treating a chronic obstructive pulomonary disease in a subject comprising administering an anti-C5 antibody to the subject afflicted with a chronic obstructive pulomonary disease.
8. A method of reducing inflammation in a subject comprising administering an anti-C5 antibody to a subject having established airway inflammation or a subject that has experienced previous asthmatic symptoms.

Samsung Bioepis Exhibit No. 1002
Page 8270

9. A method of treating a subject having established airway inflammation or a subject that has experienced previous asthmatic symptoms comprising administering an effective bronchial-dilating amount of an anti-C5 antibody.

10. A method as in claim 8 or 9 wherein the step of administering comprises administering the anti-C5 antibody during an asthma attack.

11. A method as in any of claims 1-9 wherein the subject is a human.

12. A method as in any of claims 1-9 wherein the step of administering an anti-C5 antibody comprises administering an anti-C5 antibody that inhibits the conversion of complement component C5 into C5a and C5b.

13. A method as in any of claims 1-9 wherein the step of administering an anti-C5 antibody comprises administering an anti-C5 antibody that binds to human complement component C5a.

14. A method as in any of claims 1-9 wherein the step of administering an anti-C5 antibody to human complement component C5b9.

15. A method as in any of claims 1-9 wherein the step of administering an anti-C5 antibody comprises administering an anti-C5 antibody selected from the group consisting of h5G1.1, h5G1.1-scFv and functional fragments of h5G1.1.

16. A method as in any of claims 1-9 wherein the step of administering an anti-C5 antibody comprises administering an anti-C5 antibody that is an antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to the alpha chain of human

30

Samsung Bioepis Exhibit No. 1002
Page 8271

complement component C5, wherein the antibody 1) inhibits complement activation in a human body fluid; 2) inhibits the binding of purified human complement component C5 to C5 convertase.

17. A method as in any of claims 1-9 wherein the wherein the step of administering an anti-C5 antibody comprises administering an anti-C5 antibody as an aerosol.

18. A method as in any of claims 1-9 wherein the step of administering an anti-C5 antibody comprises administering an anti-C5 antibody that via a method selected from the group consisting of intravenous infusion by injection and subcutaneous injection.

19. A method as in any of claims 1-9 wherein the step of administering an anti-C5 antibody comprises administering an anti-C5 antibody in combination with a member selected from the group consisting of steroids, anti-IgE antibodies, anti-IL-4 antibodies, anti-IL-5 antibodies, β2 adreno receptor agonists, leukotriene inhibitors, 5 Lipoxygenase inhibitors, β2 adreno receptor agonists, PDE inhibitors, CD23 antagonists, IL 13 antagonists, cytokine release inhibitors, histamine H1 receptor antagonists, anti-histamines and histamine release inhibitors.

20. A method for treating a subject having or susceptible to asthma comprising administering at least one member selected from the group consisting of steroids, anti-IgE antibodies, anti-IL-4 antibodies, anti-IL-5 antibodies, β2 adreno receptor agonists, leukotriene inhibitors, 5 Lipoxygenase inhibitors, β2 adreno receptor agonists, PDE inhibitors,

Samsung Bioepis Exhibit No. 1002
Page 8272

CD23 antagonists, IL 13 antagonists, cytokine release inhibitors, histamine H1 receptor antagonists, anti-histamines and histamine release inhibitors in combination with an anti-C5 antibody.

21. A method of treating asthma comprising administering an anti-C5 antibody to the lungs of a subject without substantially reducing systemic complement activity in the subject.

22. A method of treating asthma in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components, compounds which block the activity of one or more complement components and compounds which block the engagement of complement component receptors.

23. A method as in claim 22 wherein the step of administering comprises administering an anti-C5a receptor antibody.

24. A method of reducing inflammation in a subject comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components, compounds which block the activity of one or more complement components and compounds which block the engagement of complement component receptors.

Samsung Bioepis Exhibit No. 1002
Page 8273

25. A method as in claim 24 wherein the step of administering comprises administering the anti-C5 antibody during an asthma attack.

26. A method as in claim 24 wherein the step of administering comprises administering an anti-C5a receptor antibody.

27. A method of treating a subject having established airway inflammation or a subject that has experienced previous asthmatic symptoms comprising administering a compound to the subject, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components, compounds which block the activity of one or more complement components and compounds which block the engagement of complement component receptors.

28. A method as in claim 27 wherein the step of administering comprises administering the anti-C5 antibody during an asthma attack.

29. A method as in claim 27 wherein the step of administering comprises administering an anti-C5a receptor antibody.

30. A method as in any of claims 22, 24 or 27 wherein the step of administering a compound comprises administering one or more antibodies directed against a compound selected from the group consisting of complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2.

31. A method as in any of claims 22, 24 or 27 wherein the step of administering a compound comprises administering one or more

Samsung Bioepis Exhibit No. 1002
Page 8274

compounds selected from the group consisting of soluble CR1, soluble LEX-CR1, soluble MCP, soluble DAF, soluble CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

32. A method of preventing asthma attacks comprising prophylactically administering a compound to a subject having established airway inflammation or a subject that has experienced previous asthmatic symptoms, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components, compounds which block the activity of one or more complement components and compounds which block the engagement of complement component receptors.

33. A method as in claim 32 wherein the step of administering comprises administering an anti-C5a receptor antibody.

34. A method of reducing the severity of an asthma attack comprising administering a compound to a subject having an asthma attack, the compound being selected from the group consisting of compounds which bind to one or more complement components, compounds which block the generation of one or more complement components, compounds which block the activity of one or more complement components and compounds which block the engagement of complement component receptors.

35. A method as in claim 34 wherein the step of administering comprises administering an anti-C5a receptor antibody.

Samsung Bioepis Exhibit No. 1002
Page 8275

36. A method of reducing inflammation in the lungs of an asthma patient comprising the step of administering to a subject having or susceptible to asthma one or more compounds selected from the group consisting of soluble CR1, soluble LEX-CR1, soluble MCP, soluble DAF, soluble CD59, Factor H, cobra venom factor, FUT-175, complestatin, K76 COOH and antibodies directed against a compound selected from the group consisting of complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2.

37. A method as in claim 36 wherein the compound administered reduces the release or production of one or more inflammatory mediators in the airways of the subject.

38. A method as in claim 36 wherein the step of administering comprises administering a compound that acts at the cellular level to reduce the production or release of an inflammatory mediator.

39. A method as in claim 36 wherein the step of administering comprises administering a compound that can interact with an active form of the inflammatory mediator to prevent the inflammatory effects associated therewith.

40. A method as in claim 36 wherein the step of administering a compound comprises administering one or more antibodies directed against a compound selected from the group consisting of complement components C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, Factor D, Factor B, Factor P, MBL, MASP-1, and MASP-2.

35

Samsung Bioepis Exhibit No. 1002
Page 8276

41. A method as in claim 36 wherein the step of administering a compound comprises administering one or more compounds selected from the group consisting of CR1, LEX-CR1, MCP, DAF, CD59, Factor H, cobra venom factor, FUT-175, complestatin, and K76 COOH.

42. A method as in claim 36 wherein the step of administering a compound comprises administering the compound in combination with a member selected from the group consisting of steroids, anti-IgE antibodies, anti-IL-4 antibodies, anti-IL-5 antibodies, β2 receptor agonists, leukotriene inhibitors, 5 Lipoxygenase inhibitors, β2 adreno receptor agonists, PDE inhibitors, CD23 antagonists, IL 13 antagonists, cytokine release inhibitors, histamine H1 receptor antagonists, anti-histamines and histamine release inhibitors.

43. A method as in claim 36 wherein the step of administering a compound comprises administering an anti-C5 antibody.

44. A method as in claim 34 wherein the step of administering comprises administering an anti-C5a receptor antibody.

Samsung Bioepis Exhibit No. 1002
Page 8277

FIG. 1

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Induction of Asthmatic Attack in Normal BALB/c Mice and Effect of Prophylactic C5 Inhibition

Group & Schedule:	day 1, 14	day 25, 29, 32	day 28, 29, 30	day 33
P. Control	Ova + Alum	135.8, 40mg/kg	1% OVA aerosol	5% OVA aerosol
Anti-C5	Ova + Alum	BB5.1, 40mg/kg	1% OVA aerosol	5% OVA aerosol
Steroid	Ova + Alum	Dex	1% OVA aerosol	5% OVA aerosol
N. Control	PBS + Alum	PBS	PBS	PBS

FIG. 2a

SUBSTITUTE SHEET (RULE 26)

Samsung Bioepis Exhibit No. 1002
Page 8279

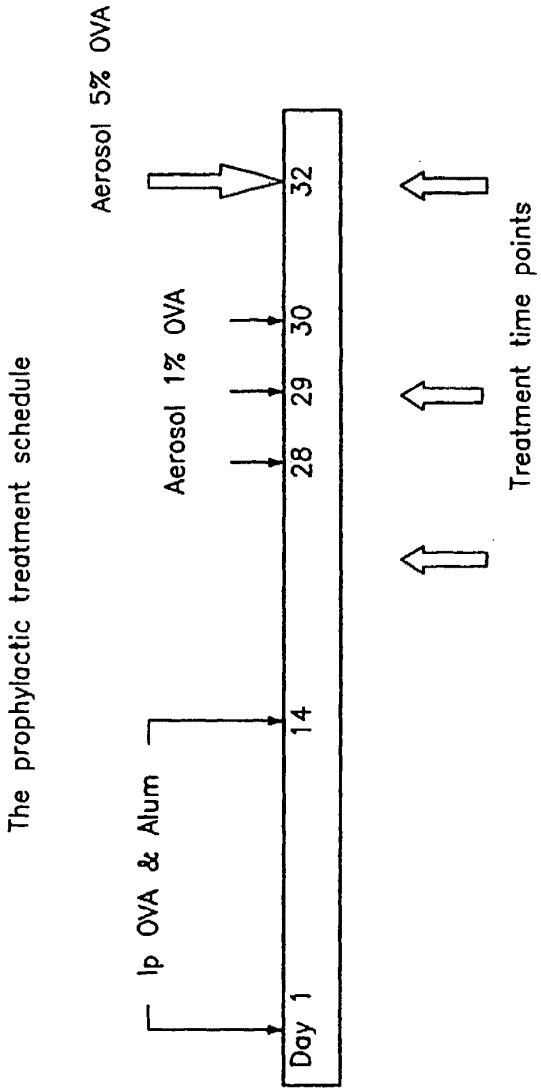


FIG. 2b

Samsung Bioepis Exhibit No. 1002
Page 8280

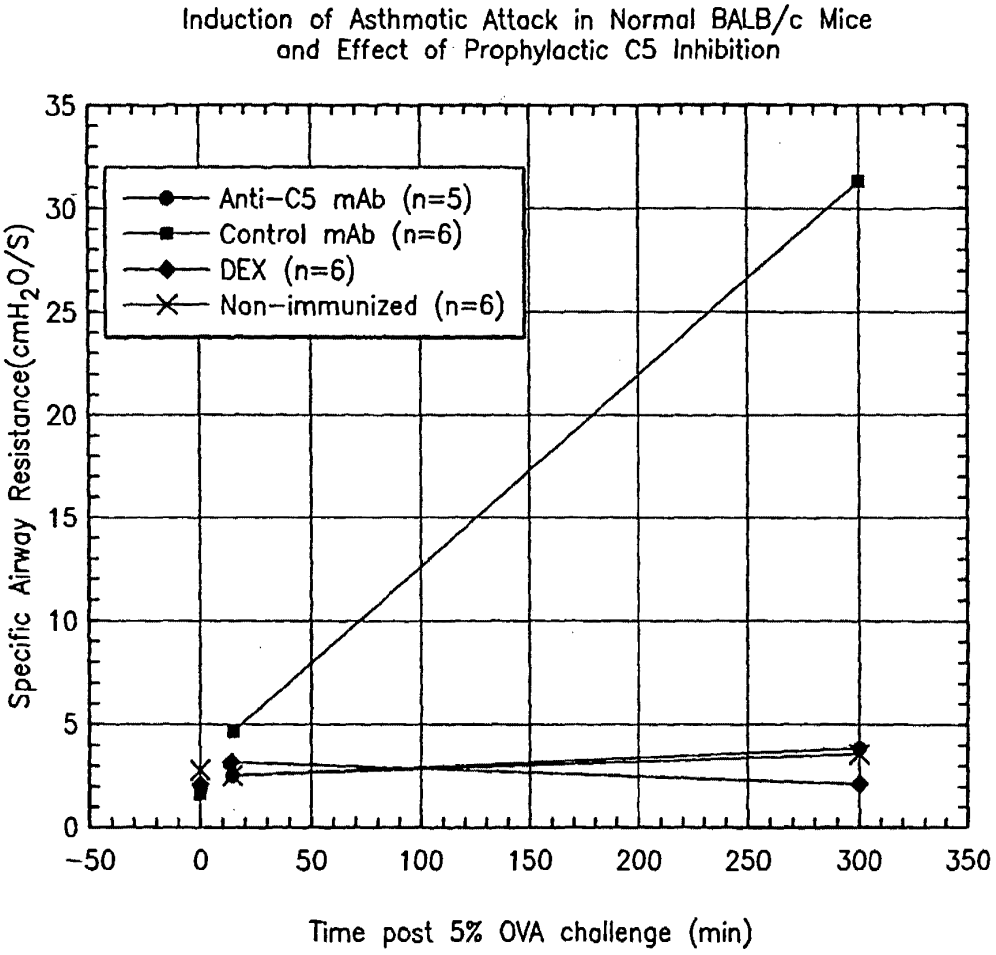


FIG. 3

Samsung Bioepis Exhibit No. 1002
Page 8281

Effect of Therapeutic C5 Inhibition (Aerosol or IV) During Asthmatic Attack

Group & Schedule:	day 1, 14	Aerosol on day 28–30 & day 32	day 35	day 35
P.Control	Ova + Alum	1% ova on day 29–30, 5% on 32	5% OVA	IV or aerosol 135.8
Anti-C5	Ova + Alum	1% ova on day 29–30, 5% on 32	5% OVA	IV or aerosol BB5.1
Steroid	Ova + Alum	1% ova on day 29–30, 5% on 32	5% OVA	IV or aerosol Dex
N.Control	PBS + Alum	PBS on day 29–30, PBS on 32	PBS	IV or aerosol PBS

FIG. 4a

Samsung Bioepis Exhibit No. 1002
Page 8282

SUBSTITUTE SHEET (RULE 26)

152

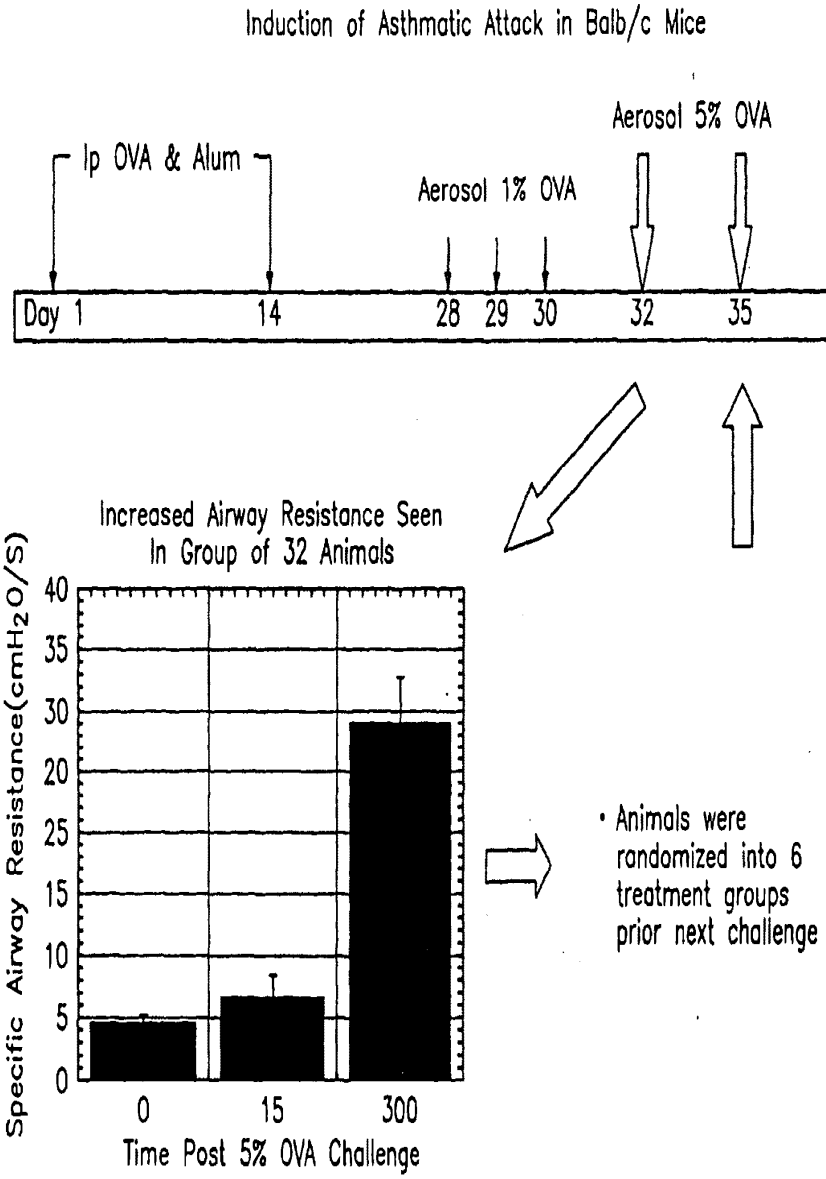


FIG. 4b

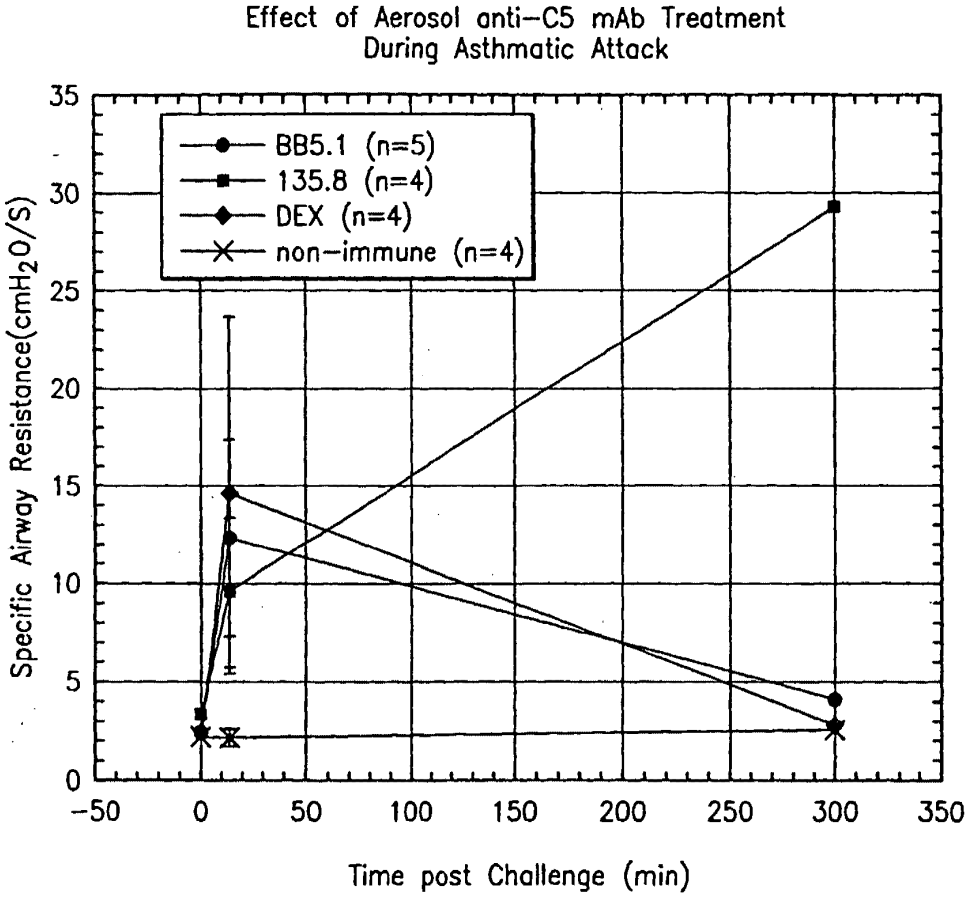


FIG. 5a

Samsung Bioepis Exhibit No. 1002
Page 8284

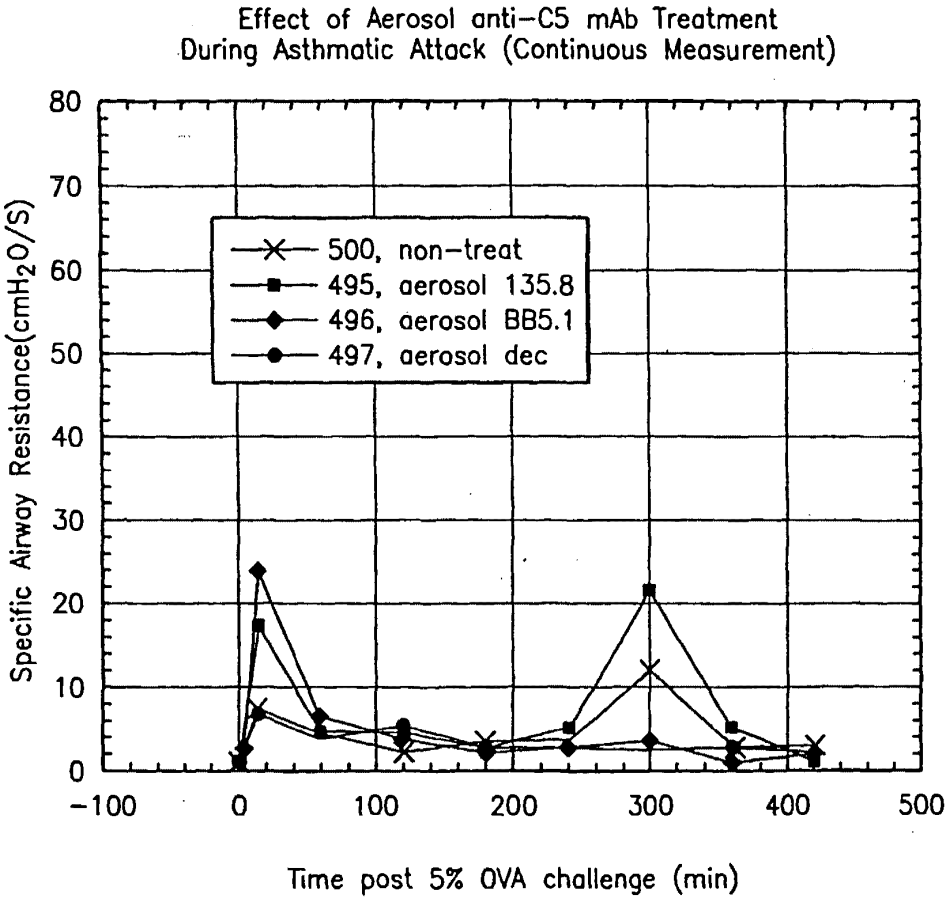

Effect of Aerosol anti-C5 mAb Treatment
During Asthmatic Attack (Continuous Measurement)
Specific Airway Resistance(cmH2O/S)
Time post 5% OVA challenge (min)
500, non-treat
495, aerosol 135.8
496, aerosol BB5.1
497, aerosol dec
80
70
60
50
40
30
20
10
0
-100
0
100
200
300
400
500

FIG. 5b

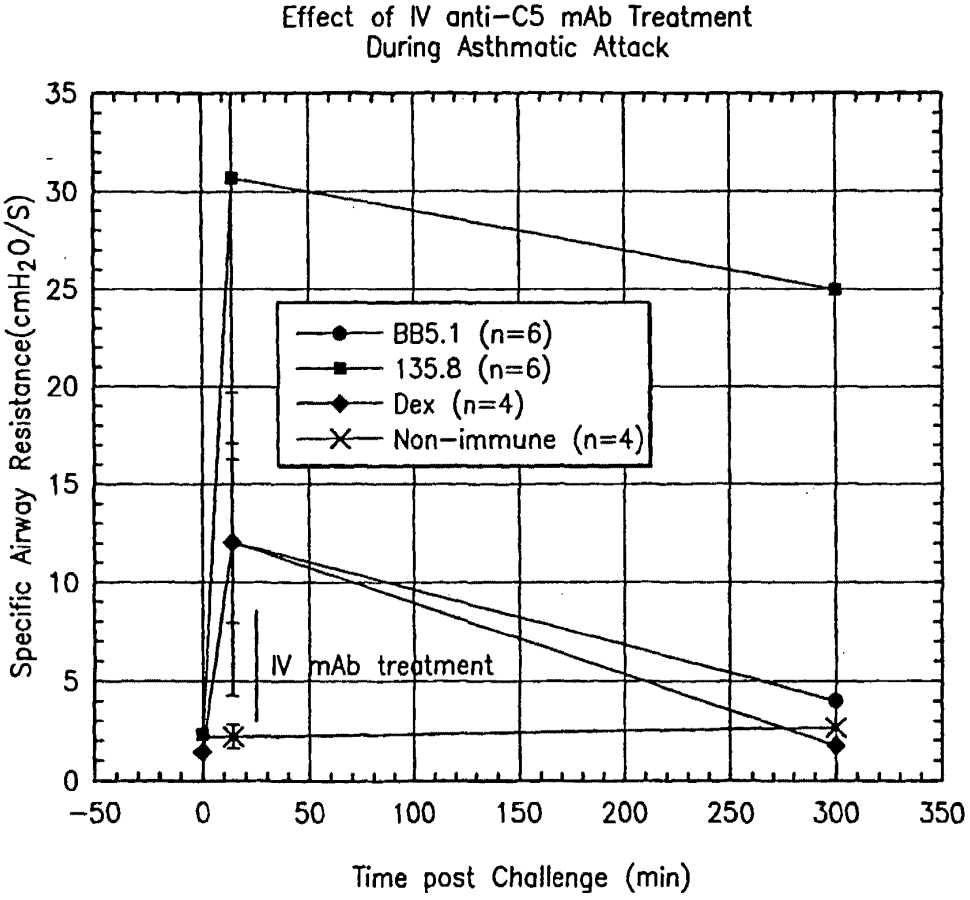


FIG. 6a

Samsung Bioepis Exhibit No. 1002
Page 8286

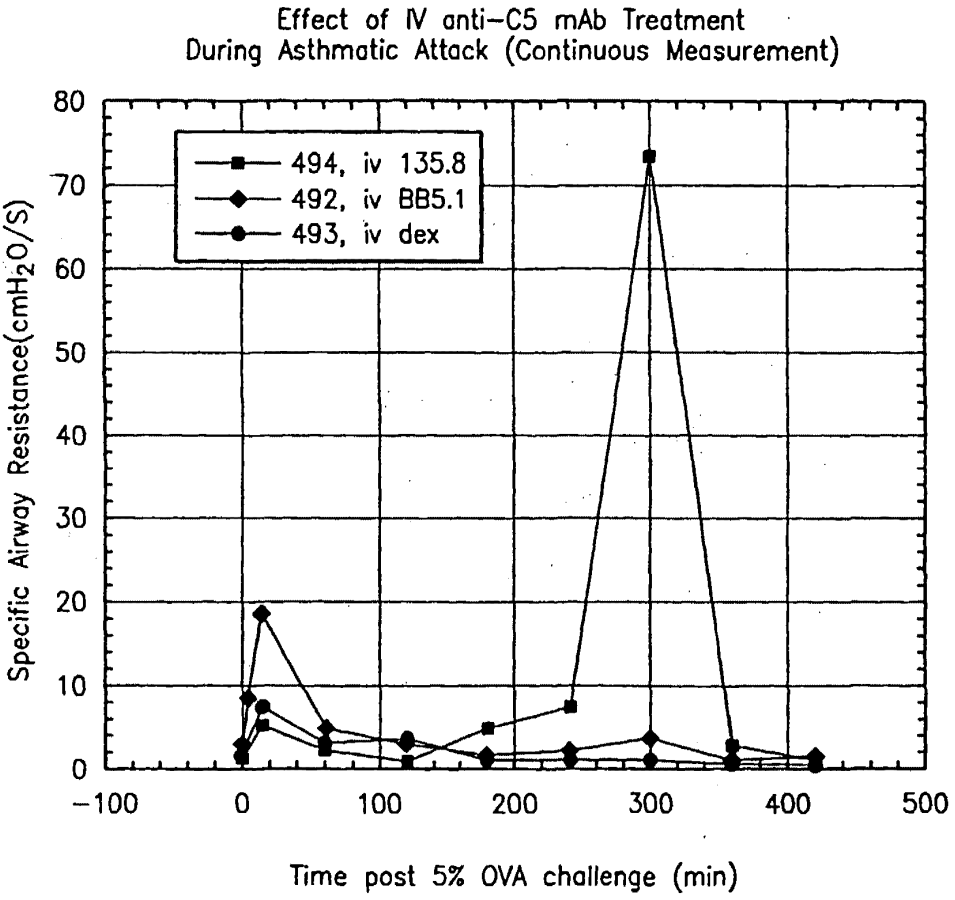


FIG. 6b

Samsung Bioepis Exhibit No. 1002
Page 8287

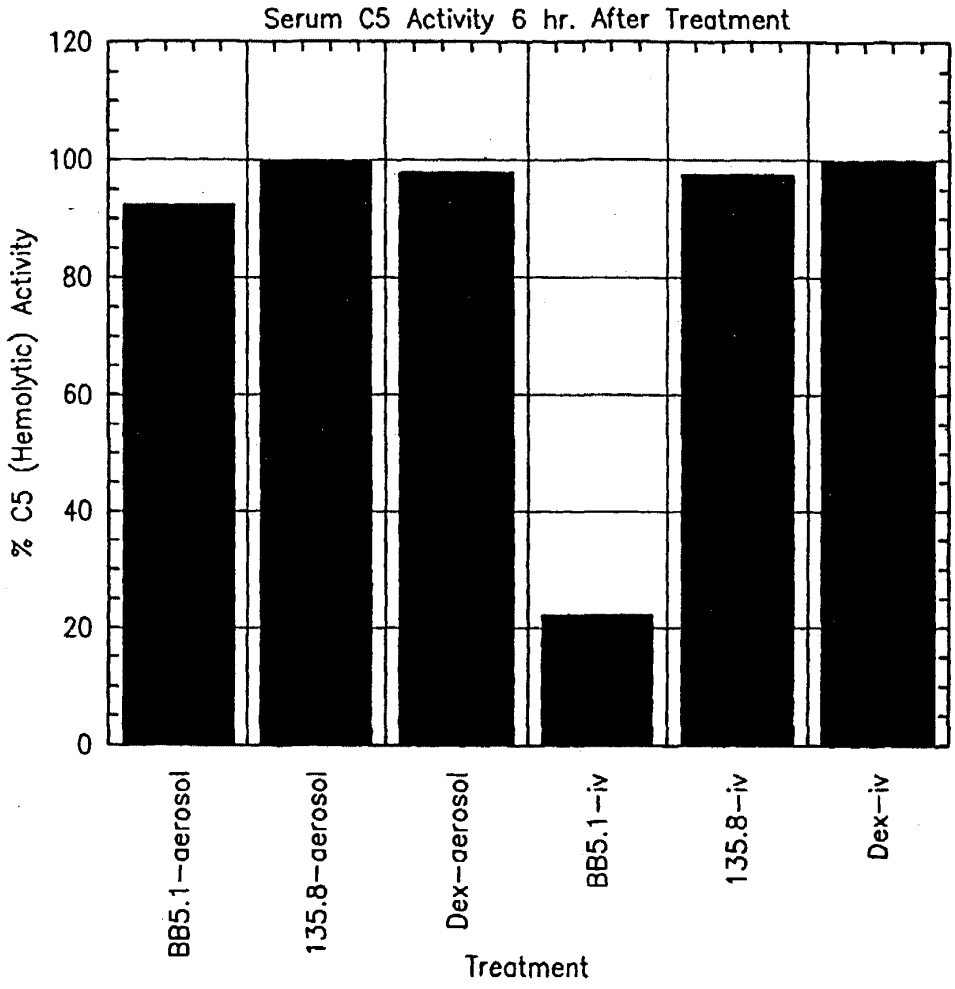


FIG. 7

Samsung Bioepis Exhibit No. 1002
Page 8288

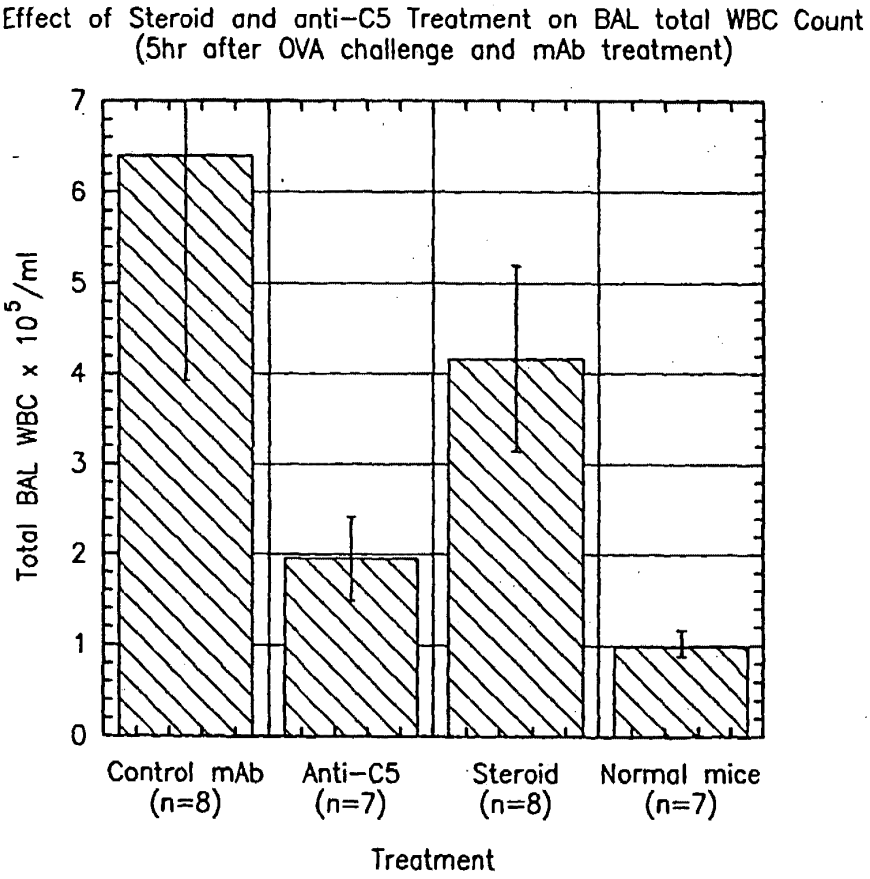


FIG. 8

Samsung Bioepis Exhibit No. 1002
Page 8289

Eosinophils are the predominant Inflammatory cells in BAL



Non-immunized

FIG. 9a



Control mAb

FIG. 9b



Steroid

FIG. 9c



Anti-C5

FIG. 9d

SUBSTITUTE SHEET (RULE 26)
159

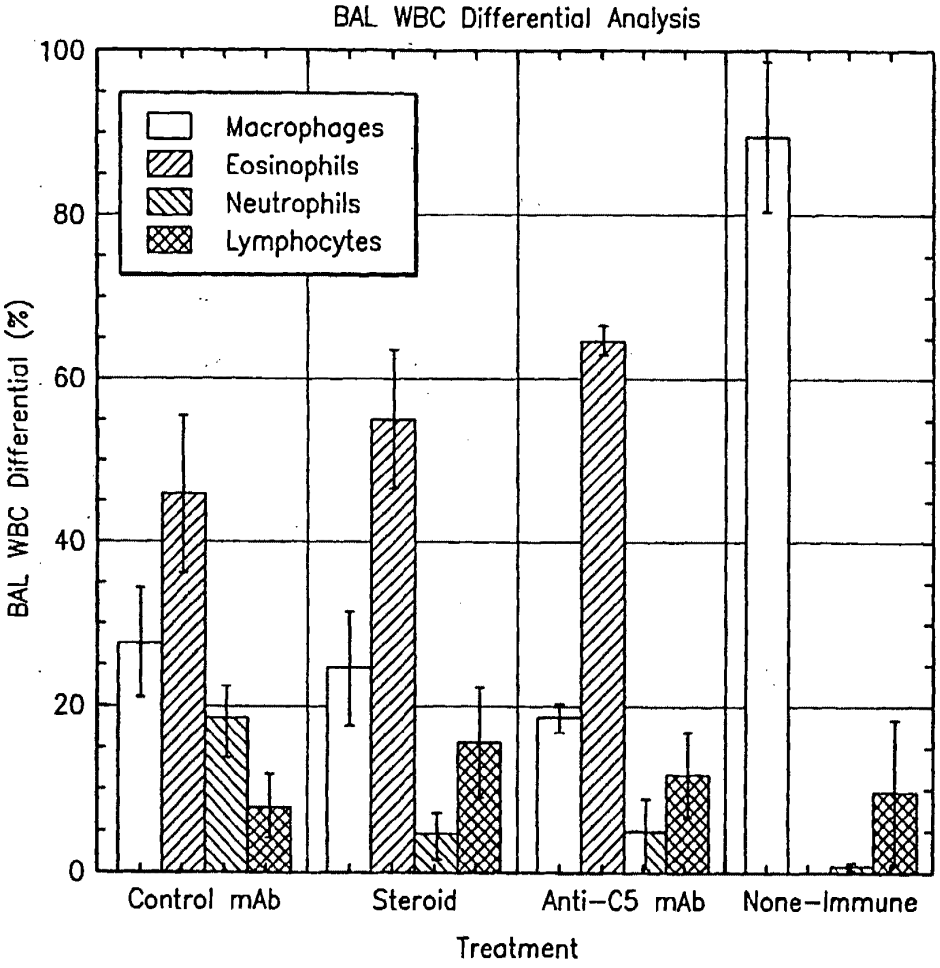

BAL WBC Differential Analysis
Macrophages
Eosinophils
Neutrophils
Lymphocytes
BAL WBC Differential (%)
100
80
60
40
20
0
Control mAb
Steroid
Anti-C5 mAb
None-Immune
Treatment

FIG. 10

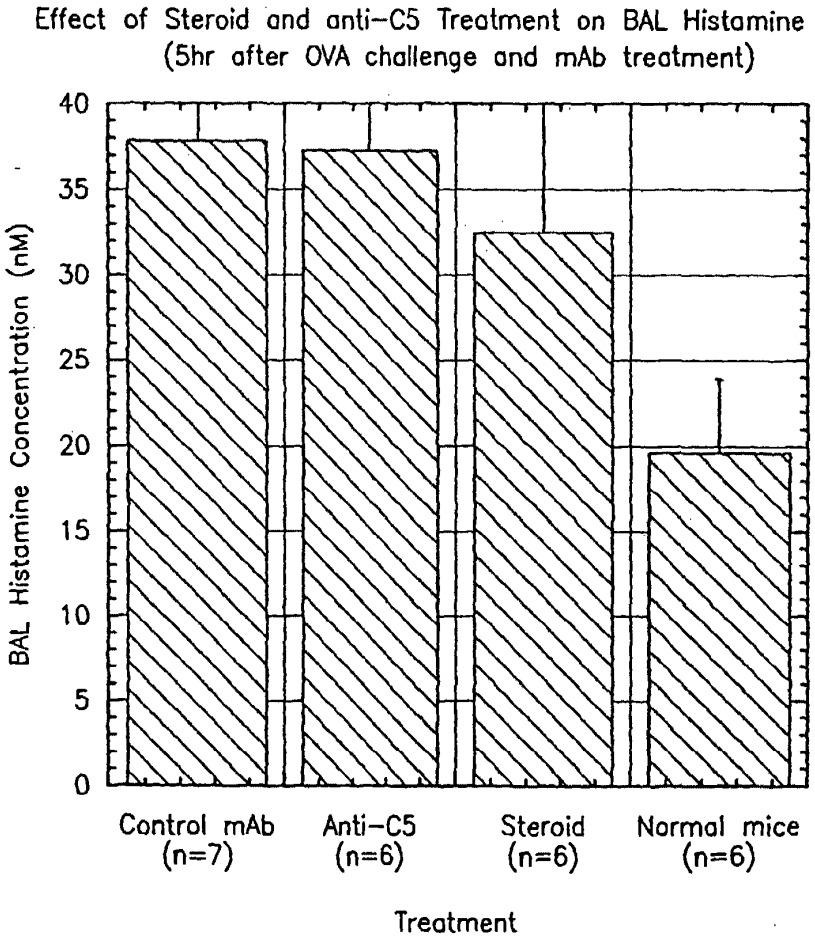

Effect of Steroid and anti-C5 Treatment on BAL Histamine Level
(5hr after OVA challenge and mAb treatment)
BAL Histamine Concentration (nM)
40
35
30
25
20
15
10
5
0
Control mAb (n=7)
Anti-C5 (n=6)
Steroid (n=6)
Normal mice (n=6)
Treatment

FIG. 11

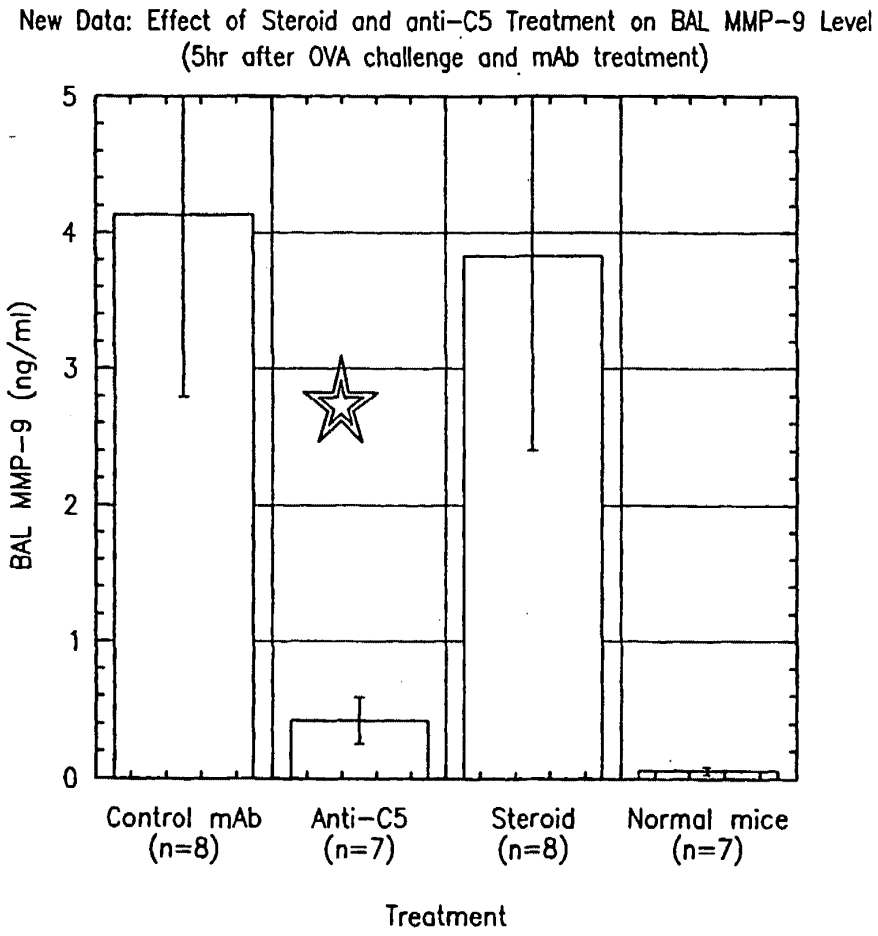


FIG. 12

Samsung Bioepis Exhibit No. 1002
Page 8293

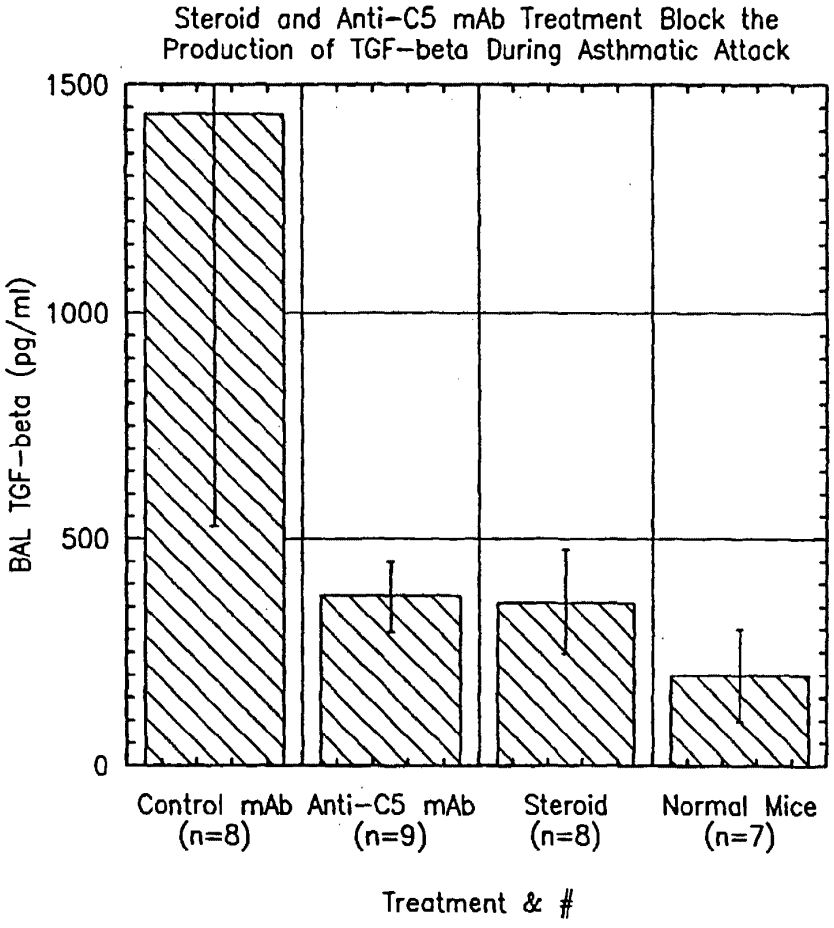


FIG. 13

Samsung Bioepis Exhibit No. 1002
Page 8294

Test the Direct & Immediate Bronchial Dilation Effect of C5 Inhibitor Or In Combination with Salbutamol During Wheezing

(Provocation with Allergen)

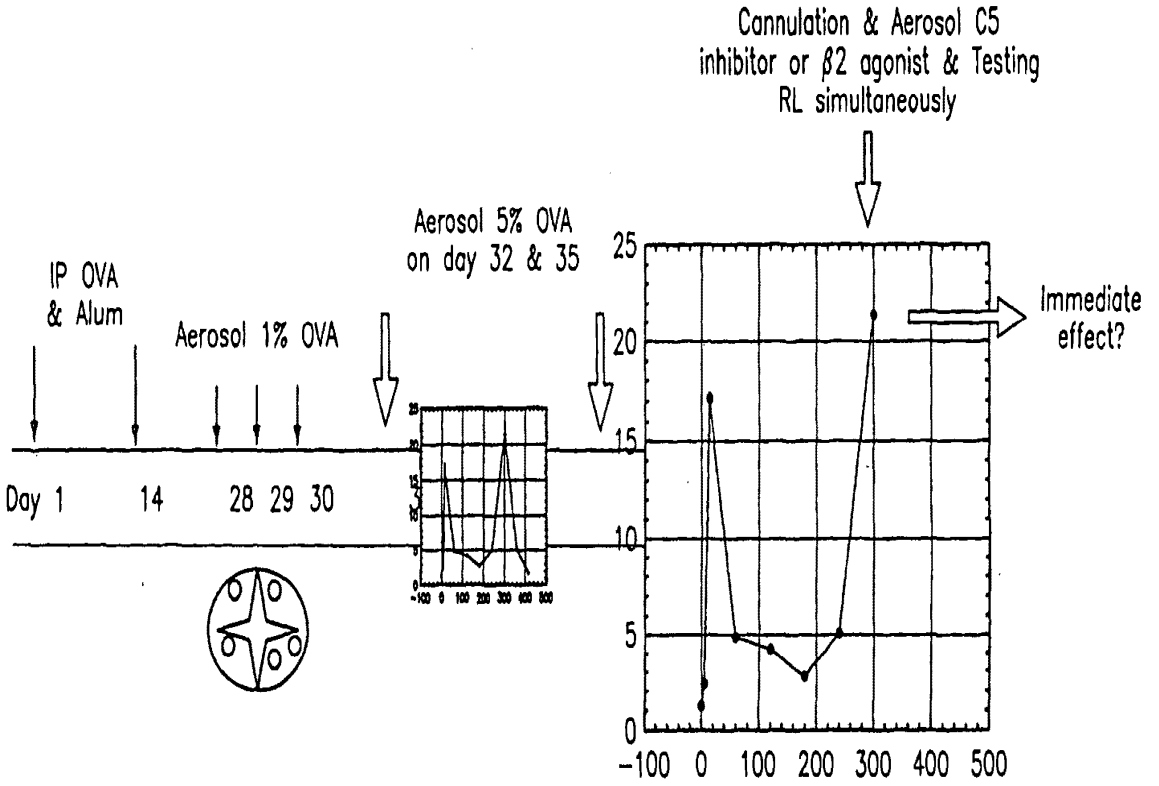


FIG. 14

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Samsung Bioepis Exhibit No. 1002
Page 8295

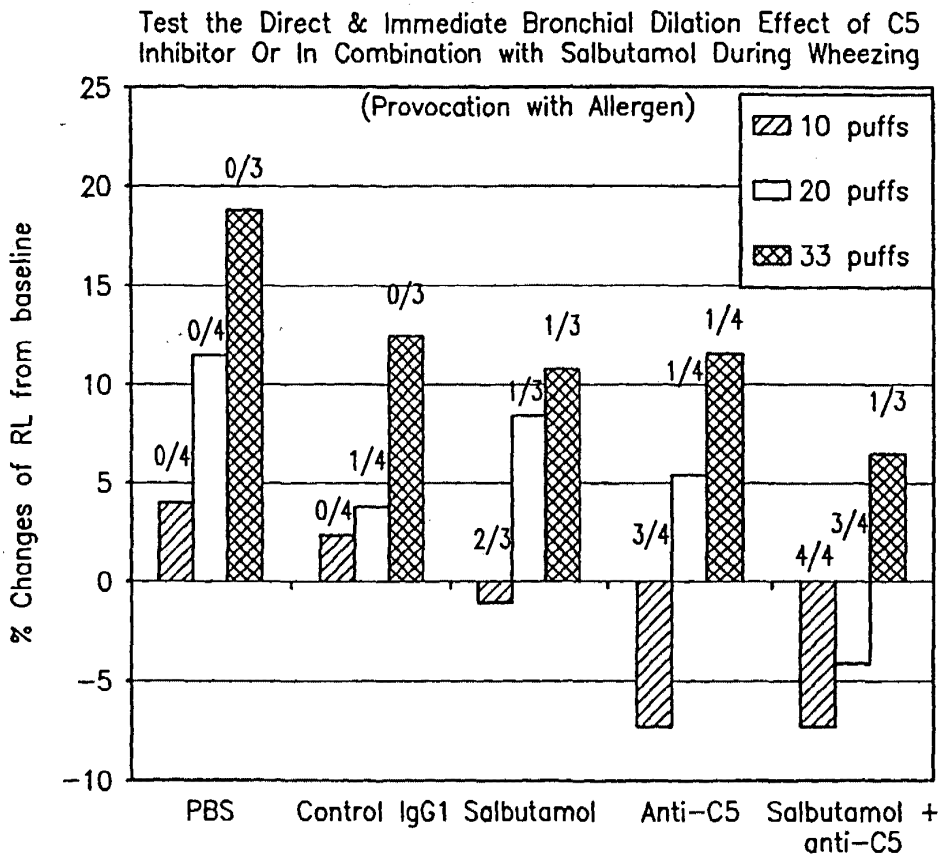


FIG. 15

Samsung Bioepis Exhibit No. 1002
Page 8296

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US03/27808

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 39/395; C07K 16/36
US CL : 424/130.1, 133.1, 141.1, 145.1, 158.1; 530/387.1, 387.3, 388.1, 388.25
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 424/130.1, 133.1, 141.1, 145.1, 158.1; 530/387.1, 387.3, 388.1, 388.25

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 6,355,245 B1 (EVANS et al.) 12 March 2002 (12 March 2002), see entire document.	1-44
Y	US 6,316,502 B1 (LAI et al.) 13 November 2001 (13.11.2001), see entire document, including column 15, lines 34-39 and column 15, line 66 - column 16, line 2).	1-44
Y	EP 0 649 468 B1 (SMITH et al.) 15 March 2000 (15.03.2000), see entire document, including page 6, line 22.	1-44

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 December 2003 (10.12.2003)	12 FEB 2004
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner for Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703)305-3230	Authorized officer Phillip Gambel Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

Samsung Bioepis Exhibit No. 1002
Page 8297

INTERNATIONAL SEARCH REPORT

PCT/US03/27808

Continuation of B. FIELDS SEARCHED Item 3:
DIALOG, BIOSIS, CA, EMBASE, MEDLINE, WEST
search terms: c5, c5a, antibod?, 5g1.1, asthma, bronchial, pulmonary, wang

Form PCT/ISA/210 (second sheet) (July 1998)

Samsung Bioepis Exhibit No. 1002
Page 8298

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(71) Applicant *(for all designated States except US)*: EPIGENESIS PHARMACEUTICALS LLC [US/US]; 7 Clarke Drive, Cranbury, NJ 08512 (US).

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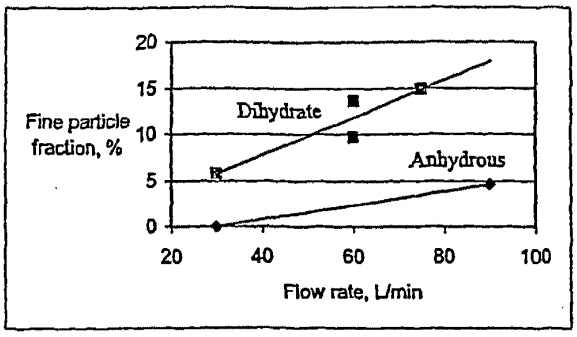
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: COMBINATION OF DEHYDROEPIANDROSTERONE OR DEHYDROEPIANDROSTERONE-SULFATE WITH AN ANTIGE ANTIBODY FOR TREATMENT OF ASTHMA OR CHRONIC OBSTRUCTIVE PULMONARY DISEASE



(57) Abstract: A pharmaceutical or veterinary composition, comprises a first active agent selected from a dehydroepiandrosterone and/or dehydroepiandrosterone-sulfate, or a salt thereof, and a second active agent comprising an anti-IgE antibody for the treatment of asthma, chronic obstructive pulmonary disease, or other respiratory diseases. The composition is provided in various formulations and in the form of a kit. The products of this patent are applied to the prophylaxis and treatment of asthma, chronic obstructive pulmonary disease, or other respiratory diseases.

WO 2005/011614 A2

Samsung Bioepis Exhibit No. 1002
Page 8299

COMBINATION OF DEHYDROEPIANDROSTERONE OR DEHYDROEPIANDROSTERONE-SULFATE WITH AN ANTI-IGE ANTIBODY FOR TREATMENT OF ASTHMA OR CHRONIC OBSTRUCTIVE PULMONARY DISEASE

BACKGROUND OF THE INVENTION

This application is a continuation-in-part of U.S. Application Serial No. 10/698,073, filed on October 29, 2003, which claims priority to the U.S. Provisional Patent Application Serial No. 60/492,231, filed on July 31, 2003, which are herein incorporated by reference in their entirety.

Field of the Invention

This invention relates to a composition comprising a non-glucocorticoid steroid including dehydroepiandrosterone (DHEA), DHEA-Sulfate, or a salt thereof, and an anti-IgE antibody. These compositions are useful in the treatment of asthma, chronic obstructive pulmonary disease (COPD), or other respiratory diseases.

Description of the Background

Respiratory ailments, associated with a variety of conditions, are extremely common in the general population. In some cases they are accompanied by inflammation, which aggravates the condition of the lungs. Respiratory ailments include asthma, chronic obstructive pulmonary disease (COPD), and other upper and lower airway respiratory diseases, such as, allergic rhinitis, Acute Respiratory Distress Syndrome (ARDS), and pulmonary fibrosis.

Asthma, for example, is one of the most common diseases in industrialized countries. In the United States it accounts for about 1% of all health care costs. An alarming increase in both the prevalence and mortality of asthma over the past decade has been reported, and asthma is predicted to be the preeminent occupational lung disease in the next decade. Asthma is a condition characterized by variable, in many instances reversible obstruction of the airways. This process is associated with lung inflammation and in some cases lung allergies. Many patients have acute episodes referred to as "asthma attacks," while others are afflicted with a chronic condition. The asthmatic process is believed to be triggered in some cases by inhalation of antigens by hypersensitive subjects. This condition is generally referred to as "extrinsic asthma." Other asthmatics have an intrinsic predisposition to the condition, which is thus referred to as "intrinsic asthma," and may be comprised of conditions of different origin, including those mediated by the adenosine receptor(s), allergic conditions mediated by an immune IgE-mediated

Samsung Bioepis Exhibit No. 1002
Page 8300

response, and others. All asthmatics have a group of symptoms, which are characteristic of this condition: episodic bronchoconstriction, lung inflammation and decreased lung surfactant. Existing bronchodilators and anti-inflammatories are currently commercially available and are prescribed for the treatment of asthma. The most common anti-inflammatories, corticosteroids, have considerable side effects but are commonly prescribed nevertheless. Most of the drugs available for the treatment of asthma are, more importantly, barely effective in a number of patients.

COPD is characterized by airflow obstruction that is generally caused by chronic bronchitis, emphysema, or both. Commonly, the airway obstruction is incompletely reversible but 10-20% of patients do show some improvement in airway obstruction with treatment. In chronic bronchitis, airway obstruction results from chronic and excessive secretion of abnormal airway mucus, inflammation, bronchospasm, and infection. Chronic bronchitis is also characterized by chronic cough, mucus production, or both, for at least three months in at least two successive years where other causes of chronic cough have been excluded. In emphysema, a structural element (elastin) in the terminal bronchioles is destroyed leading to the collapse of the airway walls and inability to exhale "stale" air. In emphysema there is permanent destruction of the alveoli. Emphysema is characterized by abnormal permanent enlargement of the air spaces distal to the terminal bronchioles, accompanied by destruction of their walls and without obvious fibrosis. COPD can also give rise to secondary pulmonary hypertension. Secondary pulmonary hypertension itself is a disorder in which blood pressure in the pulmonary arteries is abnormally high. In severe cases, the right side of the heart must work harder than usual to pump blood against the high pressure. If this continues for a long period, the right heart enlarges and functions poorly, and fluid collects in the ankles (edema) and belly. Eventually the left heart begins to fail. Heart failure caused by pulmonary disease is called *cor pulmonale*.

COPD characteristically affects middle aged and elderly people, and is one of the leading causes of morbidity and mortality worldwide. In the United States it affects about 14 million people and is the fourth leading cause of death, and the third leading cause for disability in the United States. Both morbidity and mortality, however, are rising. The estimated prevalence of this disease in the United States has risen by 41% since 1982, and age adjusted death rates rose by 71% between 1966 and 1985. This contrasts with the decline over the same period in age-adjusted mortality from all causes (which fell by 22%), and from cardiovascular diseases (which fell by 45%). In 1998 COPD accounted for 112,584 deaths in the United States.

Samsung Bioepis Exhibit No. 1002
Page 8301

COPD, however, is preventable, since it is believed that its main cause is exposure to cigarette smoke. Long-term smoking is the most frequent cause of COPD. It accounts for 80 to 90% of all cases. A smoker is 10 times more likely than a non-smoker to die of COPD. The disease is rare in lifetime non-smokers, in whom exposure to environmental tobacco smoke will explain at least some of the airways obstruction. Other proposed etiological factors include airway hyper responsiveness or hypersensitivity, ambient air pollution, and allergy. The airflow obstruction in COPD is usually progressive in people who continue to smoke. This results in early disability and shortened survival time. Smoking cessation shows the rate of decline to that of a non-smoker but the damage caused by smoking is irreversible. Other risk factors include: heredity, second-hand smoke, exposure to air pollution at work and in the environment, and a history of childhood respiratory infections. The symptoms of COPD include: chronic coughing, chest tightness, shortness of breath at rest and during exertion, an increased effort to breathe, increased mucus production, and frequent clearing of the throat.

There is very little currently available to alleviate symptoms of COPD, prevent exacerbations, preserve optimal lung function, and improve daily living activities and quality of life. Many patients will use medication chronically for the rest of their lives, with the need for increased doses and additional drugs during exacerbations. Medications that are currently prescribed for COPD patients include: fast-acting β2-agonists, anticholinergic bronchodilators, long-acting bronchodilators, antibiotics, and expectorants. Amongst the currently available treatments for COPD, short term benefits, but not long term effects, were found on its progression, from administration of anti-cholinergic drugs, β2 adrenergic agonists, and oral steroids. Oral steroids are only recommended for acute exacerbations with long term use contributing to excess mortality and morbidity.

Short and long acting inhaled β2 adrenergic agonists achieve short-term bronchodilation and provide some symptomatic relief in COPD patients, but show no meaningful maintenance effect on the progression of the disease. Short acting β2 adrenergic agonists improve symptoms in subjects with COPD, such as increasing exercise capacity and produce some degree of bronchodilation, and even an increase in lung function in some severe cases. The maximum effectiveness of the newer long acting inhaled, β2 adrenergic agonists was found to be comparable to that of short acting β2 adrenergic agonists. Salmeterol was found to improve symptoms and quality of life, although only producing modest or no change in lung function. The use of β2-agonists can produce cardiovascular effects, such as altered pulse rate, blood pressure and electrocardiogram results. In rare cases, the use of β2-agonists can produce hypersensitivity

Samsung Bioepis Exhibit No. 1002
Page 8302

reactions, such as urticaria, angioedema, rash and oropharyngeal edema. In these cases, the use of the β2-agonist should be discontinued. Continuous treatment of asthmatic and COPD patients with the bronchodilators ipratropium bromide or fenoterol was not superior to treatment on an as-needed basis, therefore indicating that they are not suitable for maintenance treatment. The most common immediate adverse effect of β2 adrenergic agonists, on the other hand, is tremors, which at high doses may cause a fall in plasma potassium, dysrhythmias, and reduced arterial oxygen tension. The combination of a β2 adrenergic agonist with an anti-cholinergic drug provides little additional bronchodilation compared with either drug alone. The addition of ipratropium to a standard dose of inhaled β2 adrenergic agonists for about 90 days, however, produces some improvement in stable COPD patients over either drug alone. Overall, the occurrence of adverse effects with β2 adrenergic agonists, such as tremor and dysrhythmias, is more frequent than with anti-cholinergics. Thus, neither anti-cholinergic drugs nor β2 adrenergic agonists have an effect on all people with COPD; nor do the two agents combined.

Anti-cholinergic drugs achieve short-term bronchodilation and produce some symptom relief in people with COPD, but no improved long-term prognosis. Most COPD patients have at least some measure of airways obstruction that is somewhat alleviated by ipratropium bromide. "The Lung Health Study" found spirometric signs of early COPD in men and women smokers and followed them for five years. Three treatments were compared over a five year period and results show that ipratropium bromide had no significant effect on the decline in the functional effective volume of the patient's lungs whereas smoking cessation produced a slowing of the decline in the functional effective volume of the lungs. Ipratropium bromide, however, produced adverse effects, such as cardiac symptoms, hypertension, skin rashes, and urinary retention.

Theophyllines produce modest bronchodilation in COPD patients whereas they have frequent adverse effects, and a small therapeutic range. Serum concentrations of 15-20 mg/l are required for optimal effects and serum levels must be carefully monitored. Adverse effects include nausea, diarrhea, headache, irritability, seizures, and cardiac arrhythmias, occurring at highly variable blood concentrations and, in many people, even within the therapeutic range. The theophyllines' doses must be adjusted individually according to smoking habits, infection, and other treatments, which is cumbersome. Although theophyllines have been claimed to have an anti-inflammatory effect in asthma, especially at lower doses, none has been reported in COPD. The adverse effects of theophyllines and the need for frequent monitoring limit their usefulness.

Oral corticosteroids have been shown to improve the short term outcome in acute exacerbations of COPD but long term administration of oral steroid has been associated with

Samsung Bioepis Exhibit No. 1002
Page 8303

serious side effects including osteoporosis and inducing overt diabetes. Inhaled corticosteroids have been found to have no real short-term effect on airway hyper-responsiveness to histamine. In two studies of 3 year treatment with inhaled fluticasone, moderate and severe exacerbations were significantly reduced as well as a modest improvement in the quality of life without affecting pulmonary function. COPD patients with more reversible disease seem to benefit more from treatment with inhaled fluticasone.

Mucolytics have a modest beneficial effect on the frequency and duration of exacerbations but an adverse effect on lung function. Neither N-acetylcysteine nor other mucolytics, however, have a significant effect in people with severe COPD (functional effective volume<50%) in spite of evidencing greater reductions in frequency of exacerbation. N-acetylcysteine produced gastrointestinal side effects. Long-term oxygen therapy administered to hypoxaemic COPD and congestive cardiac failure patients, had little effect on their rates of death for the first 500 days or so, but survival rates in men increased afterwards and remained constant over the next five years. In women, however, oxygen decreased the rates of death throughout the study. Continuous oxygen treatment of hypoxemic COPD patients for 19.3 years decreased overall risk of death. To date, however, only life style changes, smoking cessation and long term treatment with oxygen (in hypoxaemics), have been found to alter the long-term course of COPD.

Antibiotics are also often given at the first sign of a respiratory infection to prevent further damage and infection in diseased lungs. Expectorants help loosen and expel mucus secretions from the airways, and may help make breathing easier. In addition, other medications may be prescribed to manage conditions associated with COPD. These may include: diuretics (which are given as therapy to avoid excess water retention associated with right-heart failure), digitalis (which strengthens the force of the heartbeat), and cough suppressants. This latter list of medications help alleviate symptoms associated with COPD but do not treat COPD. Thus, there is very little currently available to alleviate symptoms of COPD, prevent exacerbations, preserve optimal lung function, and improve daily living activities and quality of life.

Acute Respiratory Distress Syndrome (ARDS), or stiff lung, shock lung, pump lung and congestive atelectasis, is believed to be caused by fluid accumulation within the lung which, in turn, causes the lung to stiffen. The condition is triggered within 48 hours by a variety of processes that injure the lungs such as trauma, head injury, shock, sepsis, multiple blood transfusions, medications, pulmonary embolism, severe pneumonia, smoke inhalation, radiation, high altitude, near drowning, and others. In general, ARDS occurs as a medical emergency and may be caused by other conditions that directly or indirectly cause the blood vessels to "leak"

Samsung Bioepis Exhibit No. 1002
Page 8304

fluid into the lungs. In ARDS, the ability of the lungs to expand is severely decreased and produces extensive damage to the air sacs and lining or endothelium of the lung. ARDS' most common symptoms are labored, rapid breathing, nasal flaring, cyanosis blue skin, lips and nails caused by lack of oxygen to the tissues, anxiety, and temporarily absent breathing. A preliminary diagnosis of ARDS may be confirmed with chest X-rays and the measurement of arterial blood gas. In some cases ARDS appears to be associated with other diseases, such as acute myelogenous leukemia, with acute tumor lysis syndrome (ATLS) developed after treatment with, e.g. cytosine arabinoside. In general, however, ARDS appears to be associated with traumatic injury, severe blood infections such as sepsis, or other systemic illness, high dose radiation therapy and chemotherapy, and inflammatory responses which lead to multiple organ failure, and in many cases death. In premature babies ("preemies"), neither the lung tissue nor the surfactant is fully developed. When Respiratory Distress Syndrome (RDS) occurs in preemies, it is an extremely serious problem. Preterm infants exhibiting RDS are currently treated by ventilation and administration of oxygen and surfactant preparations. When preemies survive RDS, they frequently develop bronchopulmonary dysplasia (BPD), also called chronic lung disease of early infancy, which is often fatal.

Allergic rhinitis afflicts one in five Americans, accounting for an estimated $4 to 10 billion in health care costs each year, and occurs at all ages. Because many people mislabel their symptoms as persistent colds or sinus problems, allergic rhinitis is probably underdiagnosed. Typically, IgE combines with allergens in the nose to produce chemical mediators, induction of cellular processes, and neurogenic stimulation, causing an underlying inflammation. Symptoms include ocular and nasal congestion, discharge, sneezing, and itching. Over time, allergic rhinitis sufferers often develop sinusitis, otitis media with effusion, and nasal polyposis. Approximately 60% of patients with allergic rhinitis also have asthma and flares of allergic rhinitis aggravate asthma. Degranulation of mast cells results in the release of preformed mediators that interact with various cells, blood vessels, and mucous glands to produce the typical rhinitis symptoms. Most early- and late-phase reactions occur in the nose after allergen exposure. The late-phase reaction is seen in chronic allergic rhinitis, with hypersecretion and congestion as the most prominent symptoms. Repeated exposure causes a hypersensitivity reaction to one or many allergens. Sufferers may also become hyperreactive to nonspecific triggers such as cold air or strong odors. Nonallergic rhinitis may be induced by infections, such as viruses, or associated with nasal polyps, as occurs in patients with aspirin idiosyncrasy.

Medical conditions such as pregnancy or hypothyroidism and exposure to occupational

Samsung Bioepis Exhibit No. 1002
Page 8305

factors or medications may cause rhinitis. The so-called NARES syndrome (Nonallergic Rhinitis with Eosinophilia Syndrome) is a non-allergic type of rhinitis associated with eosinophils in the nasal secretions, which typically occurs in middle-age and is accompanied by some loss of sense of smell. Treatment of allergic and non-allergic rhinitis is unsatisfactory. Self-administered saline improves nasal stuffiness, sneezing, and congestion and usually causes no side effects and it is, thus, the first treatment tried in pregnant patients. Saline sprays are generally used to relieve mucosal irritation or dryness associated with various nasal conditions, minimize mucosal atrophy, and dislodge encrusted or thickened mucus. If used immediately before intranasal corticosteroid dosing, saline sprays may help prevent drug-induced local irritation. Anti-histamines such as terfenadine and astemizole are also employed to treat allergic rhinitis; however, use of antihistamines have been associated with a ventricular arrhythmia known as Torsades de Points, usually in interaction with other medications such as ketoconazole and erythromycin, or secondary to an underlying cardiac problem. Loratadine, another non-sedating anti-histamine, and cetirizine have not been associated with an adverse impact on the QT interval, or with serious adverse cardiovascular events. Cetirizine, however, produces extreme drowsiness and has not been widely prescribed. Non-sedating anti-histamines, e.g. Claritin, may produce some relieving of sneezing, runny nose, and nasal, ocular and palatal itching, but have not been tested for asthma or other more specific conditions. Terfenadine, loratadine and astemizole, on the other hand, exhibit extremely modest bronchodilating effects, reduction of bronchial hyper-reactivity to histamine, and protection against exercise- and antigen-induced bronchospasm. Some of these benefits, however, require higher-than-currently-recommended doses. The sedating-type anti-histamines help induce night sleep, but they cause sleepiness and compromise performance if taken during the day. When employed, anti-histamines are typically combined with a decongestant to help relieve nasal congestion. Sympathomimetic medications are used as vasoconstrictors and decongestants. The three commonly prescribed systemic decongestants, pseudoephedrine, phenylpropanolamine and phenylephrine cause hypertension, palpitations, tachycardia, restlessness, insomnia and headache. The interaction of phenylpropanolamine with caffeine, in doses of two to three cups of coffee, may significantly raise blood pressure. In addition, medications such as pseudoephedrine may cause hyperactivity in children. Topical decongestants, nevertheless, are only indicated for a limited period of time, as they are associated with a rebound nasal dilatation with overuse. Anti-cholinergic agents are given to patients with significant rhinorrhea or for specific conditions such as "gustatory rhinitis", usually caused by ingestion of spicy foods, and may have some beneficial effects on the common cold. Cromolyn,

Samsung Bioepis Exhibit No. 1002
Page 8306

for example, if used prophylactically as a nasal spray, reduces sneezing, rhinorrhea, and nasal pruritus, and blocks both early- and late-phase hypersensitivity responses, but produces sneezing, transient headache, and even nasal burning. Topical corticosteroids such as Vancenase are effective in the treatment of rhinitis, especially for symptoms of itching, sneezing, and runny nose but are less effective against nasal stuffiness. Depending on the preparation, however, corticosteroid nose sprays may cause irritation, stinging, burning, or sneezing, as well. Local bleeding and septal perforation can also occur sometimes, especially if the aerosol is not aimed properly. Topical steroids generally are more effective than cromolyn sodium in the treatment of allergic rhinitis. Immunotherapy, while expensive and inconvenient, often provides benefits, especially for inpatients who experience side effects from other medications. So-called blocking antibodies, and agents that alter cellular histamine release, eventually result in decreased IgE, along with many other favorable physiologic changes. This effect is useful in IgE-mediated diseases, e.g., hypersensitivity in atopic patients with recurrent middle ear infections.

Pulmonary fibrosis, interstitial lung disease (ILD), or interstitial pulmonary fibrosis, include more than 130 chronic lung disorders that affect the lung by damaging lung tissue, and produce inflammation in the walls of the air sacs in the lung, scarring or fibrosis in the interstitium (or tissue between the air sacs), and stiffening of the lung. Breathlessness during exercise may be one of the first symptoms of these diseases, and a dry cough may be present. Neither the symptoms nor X-rays are often sufficient to differentiate various types of pulmonary fibrosis. Some pulmonary fibrosis patients have known causes and some have unknown or idiopathic causes. The course of this disease is generally unpredictable and the disease is inevitably fatal. Its progression includes thickening and stiffening of the lung tissue, inflammation and difficult breathing. Most people may need oxygen therapy and the only treatment is lung transplantation.

Lung cancer is the most common cancer in the world. During 2003, there will be about 171,900 new cases of lung cancer (91,800 among men and 80,100 among women) in the US alone and approximately 375,000 cases in Europe. Lung cancer is the leading cause of cancer death among both men and women. There will be an estimated 157,200 deaths from lung cancer (88,400 among men and 68,800 among women) in 2003, accounting for 28% of all cancer deaths in the US alone. More people die of lung cancer than of colon, breast, and prostate cancers combined (American Cancer Society Web site, 2003, Detailed Guide: Lung Cancer: What are the Key Statistics?). Tobacco smoking is well established as the main cause of lung cancer and about 90% of cases are thought to be tobacco related. There is a clear dose-response relation between lung-cancer risk and the number of cigarettes smoked per day, degree of inhalation, and age at

Samsung Bioepis Exhibit No. 1002
Page 8307

initiation of smoking. Lifelong smokers have a lung-cancer risk 20-30 times greater than a non-smoker. However, risk of lung cancer decreases with time since smoking cessation. The relative risk of male ex-smokers decreases strongly with time since end of exposure, but does not reach the risk of non-smokers, and does not decrease as much as for female ex-smokers (Tyczynski et al., *Lancet Oncol.* 4(1):45-55 (2003).

Frequently, COPD and lung cancer are co-morbid diseases and the degree of underlying COPD may dictate whether a particular patient is a surgical candidate. For NSCLC (non small cell lung cancer), only surgery (with or without radiation therapy or adjuvant chemotherapy) is curative.

- The 1-year survival rate (the number of people who live at least 1 year after their cancer is diagnosed) for lung cancer was 42% in 1998, largely due to improvements in surgical techniques.
- The 5-year survival rate for all stages of non-small cell lung cancer combined is only 15%. For small cell lung cancer the 5-year relative survival rate is about 6%.
- For people whose NSCLC is found and treated early with surgery, before it has spread to lymph nodes or other organs, the average 5-year survival rate is about 50%. However, only 15% of people with lung cancer are diagnosed at this early, localized stage.

Clearly, there is much room for improvement in chemoprophylaxis of lung cancer as well as treatment of lung cancer.

Dehydroepiandrosterone (DHEA) (3β-hydroxyandrost-5-en-17-one) is a naturally occurring steroid secreted by the adrenal cortex with apparent chemoprotective properties. Epidemiological studies have shown that low endogenous levels of DHEA correlate with increased risk of developing some forms of cancer, such as pre-menopausal breast cancer in women and bladder cancer in both sexes. The ability of DHEA and DHEA analogues, such as DHEA-S (DHEA-sulfate), to inhibit carcinogenesis is believed to result from their uncompetitive inhibition of the activity of the enzyme glucose-6-phosphate dehydrogenase (G6PDH). G6PDH is the rate limiting enzyme of the hexose monophosphate pathway, a major source of intracellular ribose-5-phosphate and NADPH. Ribose-5-phosphate is a necessary substrate for the synthesis of both ribo- and deoxyribonucleotides. NADPH is a cofactor also involved in nucleic acid biosynthesis and the synthesis of hydroxmethylglutaryl Coenzyme A reductase (HMG CoA reductase). HMG CoA reductase is an unusual enzyme that requires two moles of NADPH for each mole of product, mevalonate, produced. Thus, it appears that HMG CoA reductase would be ultrasensitive to DHEA-mediated NADPH depletion, and that DHEA-treated cells would

Samsung Bioepis Exhibit No. 1002
Page 8308

rapidly show the depletion of intracellular pools of mevalonate. Mevalonate is required for DNA synthesis, and DHEA arrests human cells in the G1 phase of the cell cycle in a manner closely resembling that of the direct HMG CoA. Because G6PDH is required to produces mevalonic acid used in cellular processes such as protein isoprenylation and the synthesis of dolichol, a precursor for glycoprotein biosynthesis, DHEA inhibits carcinogenesis by depleting mevalonic acid and thereby inhibiting protein isoprenylation and glycoprotein synthesis. Mevalonate is the central precursor for the synthesis of cholesterol, as well as for the synthesis of a variety of non-sterol compounds involved in post-translational modification of proteins such as farnesyl pyrophosphate and geranyl pyrophosphate; and for dolichol, which is required for the synthesis of glycoproteins involved in cell-to-cell communication and cell structure. It has long been known that patients receiving steroid hormones of adrenocortical origin at pharmacologically appropriate doses show increased incidence of infectious disease. U.S. Patent No. 5,527,789 discloses a method of combating cancer by administering to a patient DHEA and ubiquinone, where the cancer is one that is sensitive to DHEA.

DHEA is a 17-ketosteroid which is quantitatively one of the major adrenocortical steroid hormones found in mammals. Although DHEA appears to serve as an intermediary in gonadal steroid synthesis, the primary physiological function of DHEA has not been fully understood. It has been known, however, that levels of this hormone begin to decline in the second decade of life (reaching 5% of the original level in the elderly.) Clinically, DHEA has been used systemically and/or topically for treating patients suffering from psoriasis, gout, hyperlipemia, and it has been administered to post-coronary patients. In mammals, DHEA has been shown to have weight optimizing and anti-carcinogenic effects, and it has been used clinically in Europe in conjunction with estrogen as an agent to reverse menopausal symptoms and also has been used in the treatment of manic depression, schizophrenia, and Alzheimer's disease. DHEA has been used clinically at 40 mg/kg/day in the treatment of advanced cancer and multiple sclerosis. Mild androgenic effects, hirsutism, and increased libido were the side effects observed. These side effects can be overcome by monitoring the dose and/or by using analogues. The subcutaneous or oral administration of DHEA to improve the host's response to infections is known, as is the use of a patch to deliver DHEA. DHEA is also known as a precursor in a metabolic pathway which ultimately leads to more powerful agents that increase immune response in mammals. That is, DHEA acts as a prodrug: it acts as an immuno-modulator when converted to androstenediol or androst-5-ene-3β,17β-diol (βAED), or androstenetriol or androst-5-ene-3β,7β,17β-triol (βAET). However, in vitro DHEA has certain lymphotoxic and suppressive effects on cell proliferation

Samsung Bioepis Exhibit No. 1002
Page 8309

prior to its conversion to βAED and/or βAET. It is, therefore, believed that the superior immunity enhancing properties obtained by administration of DHEA result from its conversion to more active metabolites.

Adenosine is a purine involved in intermediary metabolism, and may constitute an important mediator in the lung for various diseases, including bronchial asthma, COPD, CF, RDS, rhinitis, pulmonary fibrosis, and others. The potential role of its receptor was suggested by the finding that asthmatics respond to aerosolized adenosine with marked bronchoconstriction whereas normal individuals do not. An asthmatic rabbit animal model, the dust mite allergic rabbit model for human asthma, responded in a similar fashion to aerosolized adenosine with marked bronchoconstriction whereas non-asthmatic rabbits showed no response. More recent work with this animal model suggested that adenosine-induced bronchoconstriction and bronchial hyperresponsiveness in asthma may be mediated primarily through the stimulation of adenosine receptors. Adenosine has also been shown to cause adverse effects, including death, when administered therapeutically for other diseases and conditions in subjects with previously undiagnosed hyper-reactive airways. Adenosine plays a unique role in the body as a regulator of cellular metabolism. It can raise the cellular level of AMP, ADP and ATP that are the energy intermediates of the cell. Adenosine can stimulate or down regulate the activity of adenylate cyclase and hence regulate cAMP levels. cAMP, in turn, plays a role in neurotransmitter release, cellular division and hormone release. Adenosine's major role appears to be to act as a protective injury autocoid. In any condition in which ischemia, low oxygen tension or trauma occurs adenosine appears to play a role. Defects in synthesis, release, action and/or degradation of adenosine have been postulated to contribute to the over activity of the brain excitatory amino acid neurotransmitters, and hence various pathological states. Adenosine has also been implicated as a primary determinant underlying the symptoms of bronchial asthma and other respiratory diseases, the induction of bronchoconstriction and the contraction of airway smooth muscle. Moreover, adenosine causes bronchoconstriction in asthmatics but not in non-asthmatics. Other data suggest the possibility that adenosine receptors may also be involved in allergic and inflammatory responses by reducing the hyperactivity of the central dopaminergic system. It has been postulated that the modulation of signal transduction at the surface of inflammatory cells influences acute inflammation. Adenosine is said to inhibit the production of super-oxide by stimulated neutrophils. Recent evidence suggests that adenosine may also play a protective role in stroke, CNS trauma, epilepsy, ischemic heart disease, coronary by-pass, radiation exposure and inflammation. Overall, adenosine appears to regulate cellular metabolism through ATP, to act as

Samsung Bioepis Exhibit No. 1002
Page 8310

a carrier for methionine, to decrease cellular oxygen demand and to protect cells from ischemic injury. Adenosine is a tissue hormone or inter-cellular messenger that is released when cells are subject to ischemia, hypoxia, cellular stress, and increased workload, and or when the demand for ATP exceeds its supply. Adenosine is a purine and its formation is directly linked to ATP catabolism. It appears to modulate an array of physiological processes including vascular tone, hormone action, neural function, platelet aggregation and lymphocyte differentiation. It also may play a role in DNA formation, ATP biosynthesis and general intermediary metabolism. It is suggested that it regulates the formation of cAMP in the brain and in a variety of peripheral tissues. Adenosine regulates cAMP formation through two receptors A_1 and A_2. Via A_1 receptors, adenosine reduces adenylate cyclase activity, while it stimulates adenylate cyclase at A_2 receptors. The adenosine A_1 receptors are more sensitive to adenosine than the A_2 receptors. The CNS effects of adenosine are generally believed to be A_1-receptor mediated, where as the peripheral effects such as hypotension, bradycardia, are said to be A_2 receptor mediated.

A handful of medicaments have been used for the treatment of respiratory diseases and conditions, although in general they all have limitations. Amongst them are glucocorticoid steroids, leukotriene inhibitors, anti-cholinergic agents, anti-histamines, oxygen therapy, theophyllines, and mucolytics. Glucocorticoid steroids are the ones with the most widespread use in spite of their well documented side effects. Most of the available drugs are nevertheless effective in a small number of cases, and not at all when it comes to the treatment of asthma. No treatments are currently available for many of the other respiratory diseases. Theophylline, an important drug in the treatment of asthma, is a known adenosine receptor antagonist which was reported to eliminate adenosine-mediated bronchoconstriction in asthmatic rabbits. A selective adenosine A1 receptor antagonist, 8-cyclopentyl-1, 3-dipropylxanthine (DPCPX) was also reported to inhibit adenosine-mediated bronchoconstriction and bronchial hyperresponsiveness in allergic rabbits. The therapeutic and preventative applications of currently available adenosine A1 receptor-specific antagonists are, nevertheless, limited by their toxicity. Theophylline, for example, has been widely used in the treatment of asthma, but is associated with frequent, significant toxicity (gastrointestinal, cardiovascular, neurological and biological disturbances) resulting from its narrow therapeutic dose range. DPCPX is far too toxic to be useful clinically. The fact that, despite decades of extensive research, no specific adenosine receptor antagonist is available for clinical use attests to the general toxicity of these agents.

In some people, allergen exposure can cause a reaction known as the allergic response. This occurs when allergens are inhaled into the respiratory tract (nose, throat and lungs) and

Samsung Bioepis Exhibit No. 1002
Page 8311

attach to the mucous membranes. These allergens are seen by the immune system as foreign invaders and an immune response is produced as the body prepares to fight them off. During this response, T-cells (a cell type of the immune system) send a signal to B-cells (B-lymphocytes) and stimulate production of IgE antibodies - a key protein involved in the allergic cascade. IgE antibodies, specific to the allergen, are produced within a few weeks after exposure and released into the bloodstream. These IgE antibodies may attach to receptors on inflammatory cells such as mast cells. Unattached IgE antibodies remain free floating in the bloodstream. When an allergic individual is re-exposed to an allergen, cross-linking to IgE bound on the mast cells may occur. When cross-linking occurs, mast cells release chemical mediators such as histamine, prostaglandins and leukotrienes. These chemical mediators can cause inflammatory responses in the body. These inflammatory responses have been linked to asthma signs and symptoms such as bronchial constriction, coughing and wheezing.

Genentech, Inc., Novartis Pharmaceuticals Corporation, an affiliate of Novartis AG, and Tanox, Inc. announced on June 20, 2003 that the novel IgE-blocker Xolair® (Omalizumab) For Subcutaneous Use has been approved by the U.S. Food and Drug Administration (FDA). Xolair® is the first humanized therapeutic antibody for the treatment of asthma and the first approved therapy designed to target the antibody IgE. Xolair® is expected to be available by prescription beginning July 2003. Xolair® is the first humanized therapeutic antibody for the treatment of asthma and the first approved therapy designed to target the antibody IgE, a key underlying cause of the symptoms of allergy related asthma.

U.S. Patent No. 5,660,835 (and corresponding PCT publication WO 96/25935) discloses a novel method of treating asthma or adenosine depletion in a subject by administering to the subject a dehydroepiandrosterone (DHEA) or DHEA-related compound. The patent also discloses a novel pharmaceutical composition in regards to an inhalable or respirable formulation comprising DHEA or DHEA-related compounds that is in a respirable particle size.

U.S. Patent No. 5,527,789 discloses a method of combating cancer in a subject by administering to the subject a DHEA or DHEA-related compound, and ubiquinone to combat heart failure induced by the DHEA or DHEA-related compound.

U.S. Patent No. 6,087,351 discloses an in vivo method of reducing or depleting adenosine in a subject's tissue by administering to the subject a DHEA or DHEA-related compound.

U.S. Patent Application Ser. No. 10/454,061, filed June 3, 2003, discloses a method for treating COPD in a subject by administering to the subject a DHEA or DHEA-related compound.

U.S. Patent Application Ser. No. 10/462,901, filed June 17, 2003, discloses a stable dry

Samsung Bioepis Exhibit No. 1002
Page 8312

powder formulation of DHEA in a nebulizable form sealed in a container.

U.S. Patent Application Ser. No. 10/462,927, filed June 17, 2003, discloses a stable dry powder formulation of dihydrate crystal form of DHEA-S suitable for treating asthma and COPD.

The above patents and patent applications are herein incorporated by reference in their entirety.

There exists a well defined need for novel and effective therapies for treating respiratory, lung and cancer ailments that cannot presently be treated, or at least for which no therapies are available that are effective and devoid of significant detrimental side effects. This is the case of ailments afflicting the respiratory tract, and more particularly the lung and the lung airways, including respiratory difficulties, asthma, bronchoconstriction, lung inflammation and allergies, depletion or hyposecretion of surfactant, etc. Moreover, there is a definite need for treatments that have prophylctic and therapeutic applications, and require low amounts of active agents, which makes them both less costly and less prone to detrimental side effects.

Further, there is a need to better ensure patient compliance in the taking of medication, and a need to facilitate the taking of the plurality of compounds necessary for prevention or treatment of asthma, COPD, or other respiratory diseases.

SUMMARY OF THE INVENTION

The present invention provides for a composition comprising at least two active agents. A first active agent comprises a non-glucocorticoid steroid, such as an epiandrosterone (EA) or a salt thereof. A second active agent comprises an anti-IgE antibody. The composition comprises a combination of the first active agent and the second active agent. The amount of the first active agent and the amount of the second active agent in the composition is of an amount sufficient to effectively prophylactically or therapeutically treat a subject in danger of suffering or suffering from asthma, COPD, or other respiratory diseases when the composition is administered to the subject. The anti-IgE antibody is one that is capable of binding IgE in order to effectively prophylactically or therapeutically treat a subject in danger of suffering or suffering from asthma, COPD, or other respiratory diseases when the anti-IgE antibody is administered to the subject. The composition can further comprise other bioactive agents and formulation ingredients. The composition is a pharmaceutical or veterinary composition suitable for administration to a subject or patient, such as a human or a non-human animal (such as a non-human mammal).

The composition is useful for treating asthma, COPD, or other respiratory diseases for

Samsung Bioepis Exhibit No. 1002
Page 8313

which inflammation and its sequelae plays a role including conditions associated with bronchoconstriction, surfactant depletion and/or allergies.

The present invention also provides for methods for treating asthma, COPD, or other respiratory diseases comprising administering the composition to a subject in need of such treatment.

The present invention also provides for a use of the first active agent and the second active agent in the manufacture of a medicament for the prophylactic or therapeutic treatment of asthma, COPD, or other respiratory diseases described above.

The present invention also provides for a kit comprising the composition and a delivery device. The delivery device is capable of delivering the composition to the subject. Preferably, the delivery device comprises an inhaler provided with an aerosol or spray generating means that delivers particles. Preferably, the delivery is to the airway of the subject. More preferably, the delivery is to the lung or lungs of the subject. Preferably, the delivery is directly to the desired location.

The main advantage of using the compositions is the compliance by the patients in need of such prophylaxis or treatment. Respiratory diseases such as asthma or COPD are multifactorial with different manifestations of signs and symptoms for individual patients. As such, most patients are treated with multiple medications to alleviate different aspects of the disease. A fixed combination of the first active agent, such as DHEA or DHEA-S, and the second active agent, such as E-25 (omalizumab), permits more convenient yet targeted therapy for a defined patient subpopulation. Patient compliances should be improved by simplifying therapy and by focusing on each patient's unique disease attributes so that their specific symptoms are addressed in the most expeditious fashion. Further, there is the added advantage of convenience or savings in time in the administering of both the first and second active agents in one administration. This is especially true when the composition is administered to a region of the body of the subject that has the potential of discomfort, such as the composition administered to the airways of the subject. This is also especially true when the administration of the compositions to the subject is invasive.

In addition, the first active agent, such as DHEA or DHEA-S, is an anti-inflammatory agent that is most effective when it is delivered or deposited in the distal peripheral airways rather than the conducting airways, in the alveolar membranes and fine airways. Asthma and some COPD patients have conducting airways that are constricted, which limit the delivery (due to earlier deposition caused by lower particle velocity) of the first active agent, such as DHEA,

Samsung Bioepis Exhibit No. 1002
Page 8314

acting on these distal peripheral airways. Therefore, the combination of a bronchodilator drug (β2 agonist, antimuscarinic which reverses elevated tone) facilitates the delivery of an anti-inflammatory to the distal peripheral airways. Use of the combination provides an improved sustained pharmacologic effect that translates an improved disease management. The antileukotrienes reduce interstitial edema in the very small peripheral airways. This too would have the effect of increasing peripheral airway diameter and facilitate delivery of the first active agent. This is also true for antihistamines, which also reduce peripheral airways edema and facilitate distal airway delivery of the first active agent.

The drawings accompanying this patent form part of the disclosure of the invention, and further illustrate some aspects of the present invention as discussed below.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts fine particle fraction of neat micronized DHEA-S·$2H_2O$ delivered from the single-dose Acu-Breathe inhaler as a function of flow rate. Results are expressed as DHEA-S. IDL data on virtually anhydrous micronized DHEA-S are also shown in this figure where the 30 L/min result was set to zero since no detectable mass entered the impactor.

Figure 2 depicts HPLC chromatograms of virtually anhydrous DHEA-S bulk after storage as neat and lactose blend for 1 week at 50°C. The control was neat DHEA-S stored at room temperature (RT)

Figure 3 depicts HPLC chromatograms for DHEA-S·$2H_2O$ bulk after storage as neat and lactose blend for 1 week at 50°C. The control was neat DHEA-S·$2H_2O$ stored at RT.

Figure 4 depicts solubility of DHEA-S as a function of NaCl concentration at two temperatures.

Figure 5 depicts DHEA-S solubility as a function of the reciprocal sodium cation concentration at 24-25 °C.

Figure 6 depicts DHEA-S solubility as a function of the reciprocal sodium cation concentration at 7-8 °C.

Figure 7 depicts solubility of DHEA-S as a function of NaCl concentration with and without buffer at RT.

Figure 8 depicts DHEA-S solubility as a function of the reciprocal of sodium cation concentration at 24-25 °C with and without buffer.

Figure 9 depicts solution concentration of DHEA-S versus time at two storage conditions.

Figure 10 depicts solution concentration of DHEA versus time at two storage conditions.

Samsung Bioepis Exhibit No. 1002
Page 8315

Figure 11 depicts the schematic for nebulization experiments.

Figure 12 depicts mass of DHEA-S deposited in by-pass collector as a function of initial solution concentration placed in the nebulizer.

Figure 13 depicts particle size by cascade impaction for DHEA-S nebulizer solutions. The data presented are the average of all 7 nebulization experiments.

Figure 14 depicts the inhibition of HT-29 SF cells by DHEA.

Figure 15 depicts the effects of DHEA on cell cycle distribution in HT-29 SF cells.

Figures 16a and 16b depict the reversal of DHEA-induced growth inhibition in HT-29 cells.

Figure 17 depicts the reversal of DHEA-induced G_1 arrest in HT-29 SF cells.

Figure 18 depicts a sequence comparison of the differences between the light chain and heavy chain CDR domains between rhuMabe25, e426, and sequences e26 and e27. The residue numbering here is consecutive, as opposed to that of Kabat et al. These sequences are only fragments and do not show the full-length heavy and light chain residues. The variable light chain sequence of e26 and e27 is represented by SEQ ID NO:1. The variable light chain sequence of e426 is represented by SEQ ID NO:2. The variable light chain sequence of e25 is represented by SEQ ID NO:3. The variably heavy chain sequence of e27 is represented by SEQ ID NO:4. The variable heavy chain sequence of e25, e26 and e426 is represented by SEQ ID NO:5.

Figure 19 depicts the full length heavy and light chain residues of anti-IgE antibodies E25, E26 and E27. The full length variable light chain sequence of e25 is represented by SEQ ID NO:6. The full length heavy chain sequence of e25 is represented by SEQ ID NO:7. The full length light chain sequence of e26 is represented by SEQ ID NO:8. The full length heavy chain sequence of e26 is represented by SEQ ID NO:9. The full length light chain sequence of e27 is represented by SEQ ID NO:10. The full length heavy chain sequence of e27 is represented by SEQ ID NO:11.

Figure 20 depicts the F(ab) fragments of anti-IgE antibodies e26 and e27. The light chain sequence of a e26 and Fab fragment of e27 is represented by SEQ ID NO:12. The heavy chain sequence of a Fab fragment of e26 is represented by SEQ ID NO:13. The heavy chain sequence of a Fab fragment of e27 is represented by SEQ ID NO:14.

Figure 21 depicts the sFV fragments of anti-IgE antibodies e26 and e27. The sFv fragment of e26 is represented by SEQ ID NO:15. The sFv fragment of e27 is represented by SEQ ID NO:16.

Samsung Bioepis Exhibit No. 1002
Page 8316

Figure 22 depicts the $F(ab')_2$ fragments of anti-IgE antibodies e26 and e27. The light chain sequence of a $F(ab')_2$ fragment of e26 and e27 is represented by SEQ ID NO:17. The heavy chain sequence of a $F(ab')_2$ fragment of e26 is represented by SEQ ID NO:18. The heavy chain sequence of a $F(ab')_2$ fragment of e27 is represented by SEQ ID NO:19.

Figure 23 depicts certain suitable analogs of DHEA.

Figure 24 depicts certain suitable analogs of DHEA.

Figure 25 depicts certain suitable analogs of DHEA.

Figure 26 depicts suitable modifications of the C-17 ketone of DHEA.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Definitions

In the present context, the terms "adenosine" and "surfactant" depletion are intended to encompass levels that are lowered or depleted in the subject as compared to previous levels in that subject, and levels that are essentially the same as previous levels in that subject but, because of some other reason, a therapeutic benefit would be achieved in the patient by modification of the levels of these agents as compared to previous levels.

The term "airway", as used herein, means part of or the whole respiratory system of a subject that is exposed to air. The airway includes, but not exclusively, throat, tracheobronchial tree, nasal passages, sinuses, among others. The airway also includes trachea, bronchi, bronchioles, terminal bronchioles, respiratory bronchioles, alveolar ducts, and alveolar sacs.

The term "airway inflammation", as used herein, means a disease or condition related to inflammation on airway of subject. The airway inflammation may be caused or accompanied by allergy(ies), asthma, impeded respiration, cystic fibrosis (CF), Chronic Obstructive Pulmonary Diseases (COPD), allergic rhinitis (AR), Acute Respiratory Distress Syndrome (ARDS), microbial or viral infections, pulmonary hypertension, lung inflammation, bronchitis, cancer, airway obstruction, and bronchoconstriction.

The term "carrier", as used herein, means a biologically acceptable carrier in the form of a gaseous, liquid, solid carriers, and mixtures thereof, which are suitable for the different routes of administration intended. Preferably, the carrier is pharmaceutically or veterinarily acceptable.

"An effective amount" as used herein, means an amount which provides a therapeutic or prophylactic benefit.

"Other therapeutic agents" refers to any therapeutic agent is not the first or second active agent of the composition.

Samsung Bioepis Exhibit No. 1002
Page 8317

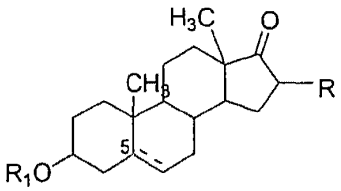
The terms "prophylaxis", as used herein, mean a prophylactic treatment made before a subject experiences a disease or a worsening of a previously diagnosed condition such that it can have a subject avoid, prevent or reduce the probability of having a disease symptom or condition related thereto. The subject can be one of increased risk of obtaining the disease or a worsening of a previously diagnosed condition.

The term "respiratory diseases", as used herein, means diseases or conditions related to the respiratory system. Examples include, but not limited to, airway inflammation, allergy(ies), impeded respiration, cystic fibrosis (CF), allergic rhinitis (AR), Acute Respiratory Distress Syndrome (ARDS), cancer, pulmonary hypertension, lung inflammation, bronchitis, airway obstruction, bronchoconstriction, microbial infection, and viral infection, such as SARS.

The terms "treat", "treating" or "therapeutic", as used herein, mean a treatment which decreases the likelihood that the subject administered such treatment will manifest symptoms of disease or other conditions.

The present invention provides for a composition comprising a first active agent comprising a non-glucocorticoid steroid, such as an epiandrosterone (EA), analogue thereof, or a salt thereof (preferably DHEA or DHEA-S), in combination with a second active agent comprising an anti-IgE antibody. The composition can further comprise a pharmaceutical or veterinarily acceptable carrier, diluent, excipient, bioactive agent or ingredient. The compositions are useful for treating asthma, COPD, or other respiratory diseases. Other respiratory diseases that the compositions are also useful for treating are lung and respiratory diseases and conditions associated with bronchoconstriction, lung inflammation and/or allergies, and lung cancer.

The first active agent is an epiandrosterone, an analogue or a pharmaceutically or veterinarily acceptable salt thereof. The epiandrosterone, an analogue or a pharmaceutically or veterinarily acceptable salt thereof is selected from a non-glucocorticoid steroid having the chemical formula



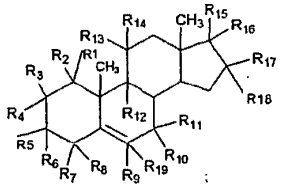
(I)

wherein the broken line represents a single or a double bond; R is hydrogen or a halogen; the H at position 5 is present in the alpha or beta configuration or the compound of chemical formula I

Samsung Bioepis Exhibit No. 1002
Page 8318

comprises a racemic mixture of both configurations; and R^1 is hydrogen or a multivalent inorganic or organic dicarboxylic acid covalently bound to the compound;

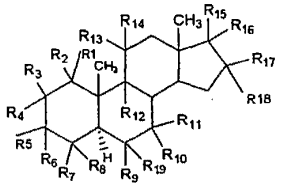
a non-glucocorticoid steroid of the chemical formula



(III)

; or

a non-glucocorticoid steroid of the chemical formula



(IV)

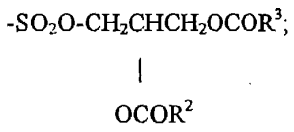
; or a combination thereof

wherein R1, R2, R3, R4. R5, R7, R8, R9, R10, R12, R13, R14 and R19 are independently H, OR, halogen, (C1-C10) alkyl or (C1-C10) alkoxy, R5 and R11 are independently OH, SH, H, halogen, pharmaceutically acceptable ester, pharmaceutically acceptable thioester, pharmaceutically acceptable ether, pharmaceutically acceptable thioether, pharmaceutically acceptable inorganic esters, pharmaceutically acceptable monosaccharide, disaccharide or oligosaccharide, spirooxirane, spirothirane, -OSO2R20, -OPOR20R21 or (C1-C10) alky, R5 and R6 taken together are =O, R10 and R11 taken together are =O; R15 is (1) H, halogen, (C1-C10) alkyl, or (C1-C10) alkoxy when R16 is -C(O)OR22, (2) H, halogen, OH or (C1-C10) alkyl when R16 is halogen, OH or (C1-C10) alkyl, (3) H, halogen, (C1-C10) alkyl, (C1-C10) alkenyl, (C1-C10) alkynyl, formyl, (C1-C10) alkanoyl or epoxy when R16 is OH, (4) OR, SH, H, halogen, pharmaceutically acceptable ester, pharmaceutically acceptable thioester, pharmaceutically acceptable ether, pharmaceutically acceptable thioether, pharmaceutically acceptable inorganic esters, pharmaceutically acceptable monosaccharide, disaccharide or oligosaccharide, spirooxirane, spirothirane, -OSO2R20 or -OPOR20R21 when R16 is H, or R15 and R16 taken together are =O; R17 and R18 are independently (1) H, -OH, halogen, (C1-C10) alkyl or -(C1-C10) alkoxy when R6 is H OR, halogen. (C1-C10) alkyl or -C(O)OR22, (2) H, (C1-C10 alkyl).amino, ((C1-C10) alkyl)n amino-(C1-C10) alkyl, (C1-C10) alkoxy, hydroxy - (C1-C10) alkyl, (C1-C10) alkoxy - (C1-C10) alkyl, (halogen)m (C1-C10) alkyl, (C1-C10) alkanoyl, formyl, (C1-C10) carbalkoxy or (C1-C10) alkanoyloxy when R15 and R16 taken together are =O, (3) R17 and R18 taken together are =O; (4) R17 or R18 taken together with the carbon to which

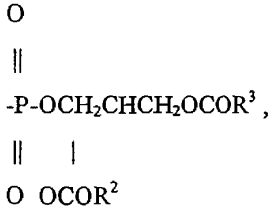
Samsung Bioepis Exhibit No. 1002
Page 8319

they are attached form a 3-6 member ring containing 0 or 1 oxygen atom; or (5) R15 and R17 taken together with the carbons to which they are attached form an epoxide ring; R20 and R21 are independently OH, pharmaceutically acceptable ester or pharmaceutically acceptable ether; R22 is H, (halogen)m (C1-C10) alkyl or (C1-C10) alkyl; n is 0, 1 or 2; and m is 1, 2 or 3; or pharmaceutically or veterinarily acceptable salts thereof.

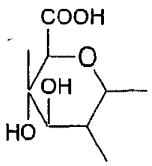
Preferably, for chemical formula (I), the multivalent organic dicarboxylic acid is SO_2OM, phosphate or carbonate, wherein M comprises a counterion. Examples of a counterion are H, sodium, potassium, magnesium, aluminum, zinc, calcium, lithium, ammonium, amine, arginine, lysine, histidine, triethylamine, ethanolamine, choline, triethanoamine, procaine, benzathine, tromethanine, pyrrolidine, piperazine, diethylamine, sulfatide



and phosphatide



wherein R^2 and R^3, which may be the same or different, are straight or branched (C_1-C_{14}) alkyl or glucuronide



The hydrogen atom at position 5 of the chemical formula I may be present in the alpha or beta configuration, or the DHEA compound may be provided as a mixture of compounds of both configurations. Compounds illustrative of chemical formula I above are included, although not exclusively, are DHEA, wherein R and R^1 are each hydrogen, containing a double bond; 16-alpha bromoepiandrosterone, wherein R is Br, R^1 is H, containing a double bond; 16-alpha-fluoro epiandrosterone, wherein R is F, R^1 is H, containing a double bond; Etiocholanolone, wherein R and R^1 are each hydrogen lacking a double bond; and dehydroepiandrosterone sulphate, wherein

Samsung Bioepis Exhibit No. 1002
Page 8320

R is H, R^1 is SO_2OM and M is a sulphatide group as defined above, lacking a double bond. Others, however, are also included. Also preferred compounds of formula I are those where R is halogen, e.g. bromo, chloro, or fluoro, where R1 is hydrogen, and where the double bond is present. A most preferred compound of formula I is 16-alpha-fluoro epiandrosterone. Other preferred compounds are DHEA and DHEA salts, such as the sulfate salt (DHEA-S).

In general, the non-glucocorticoid steroid, such as those of formulas (I), (III) and (IV), their derivatives and their salts are administered in a dosage of about 0.05, about 0.1, about 1, about 5, about 20 to about 100, about 500, about 1000, about 1500 about 1800, about 2500, about 3000, about 3600 mg/kg body weight. Other dosages, however, are also suitable and are contemplated within this patent. The first active agent of formula (I), (III) and (IV) may be made in accordance with known procedures, or variations thereof that will be apparent to those skilled in the art. See, for example, U.S. Patent No. 4,956,355; UK Patent No. 2,240,472; EPO Patent Application No. 429; 187, PCT Patent Publication No. WO 91/04030; U.S. Patent No. 5,859,000; Abou-Gharbia et al., J. Pharm. Sci. 70: 1154-1157 (1981); Merck Index Monograph No. 7710 (11th Ed. 1989), among others.

In some embodiments of the invention, the first active agent can be an epiandrosterone analog or derivative thereof. Also, prodrugs and active metabolites of epiandrosterone are encompassed by the present invention. Those of skill in the art will recognize that the compounds described herein may exhibit the phenomena of tautomerism, conformational isomerism, geometric isomerism and/or optical isomerism. It should be understood that the invention encompasses any tautomeric, conformational isomeric, optical isomeric, and/or geometric isomeric forms of the compounds having one or more of the utilities described herein, as well as mixtures of these various different forms.

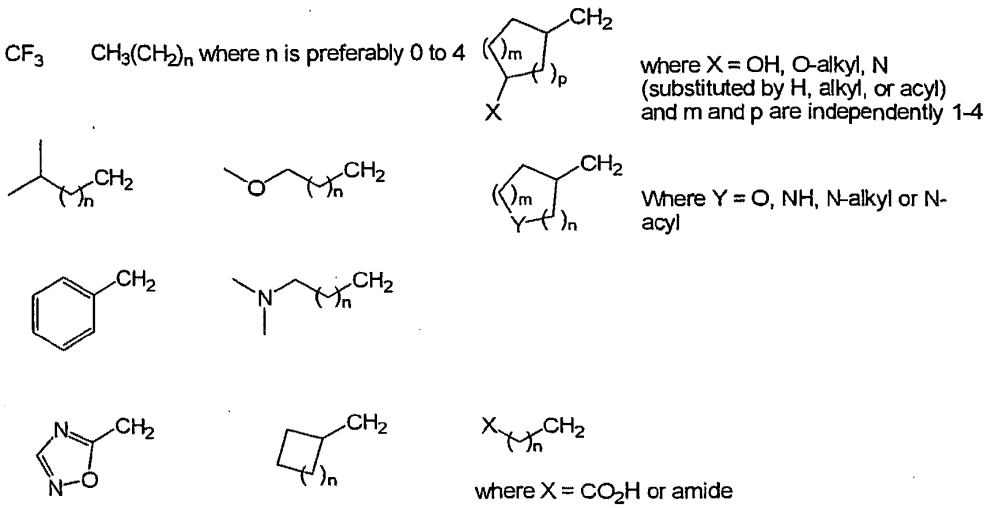
Metabolites of epiandrosterones, such as those described in the following references maybe used as the first active agent – Capillary gas chromatography of urinary steroids of terbutaline-treated asthmatic children, Chromatographia (1998), 48(1/2), 163-165; Androstenedione metabolism in human lung fibroblasts; Journal of Steroid Biochemistry (1986), 24(4), 893-7; Metabolism of androsterone and 5α-androstane-3α,17β-diol in human lung tissue and in pulmonary endothelial cells in culture, Journal of Clinical Endocrinology and Metabolism (1985), 60(2), 244-50; Testosterone metabolism by human lung tissue, Journal of Steroid Biochemistry (1978), 9(1), 29-32; Metabolism of androsterone and 5 alpha-androstane-3 alpha,17 beta-diol in human lung tissue and in pulmonary endothelial cells in culture, Journal of clinical endocrinology and metabolism (1985 Feb), 60(2), 44-50; Metabolism of

Samsung Bioepis Exhibit No. 1002
Page 8321

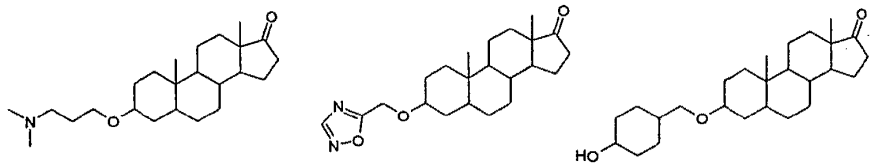
dehydroisoandrosterone and androstenedione in human pulmonary endothelial cells in culture, Journal of clinical endocrinology and metabolism (1983 May), 56(5), 930-5; Metabolism of dehydroisoandrosterone and androstenedione by the human lung in vitro, Journal of steroid biochemistry (1977 Apr), 8(4), 277-84; and Testosterone metabolism in dog lung in vitro, Steroids and lipids research (1973), 4(1), 17-23, which references are herein incorporated by reference in their entirety.

Other suitable analogs of DHEA that can be used as the first active agent are described herein. Figure 23 depicts certain suitable analogs of DHEA, including compounds of the Formulas IA, IB, IC, and ID. In the depiction of suitable R groups herein, the attachment point is indicated by a CH2 group or by an atom marked with an asterisk. R1 and R3 can be linear or branched alkyls including benzyl and optionally substituted alkyls, such as aminoalkyls, hydroxyalkyls, ethers, and carboxylic acids, and optionally substituted aryl and heteroaryls. R1 and R3 can be, for example,

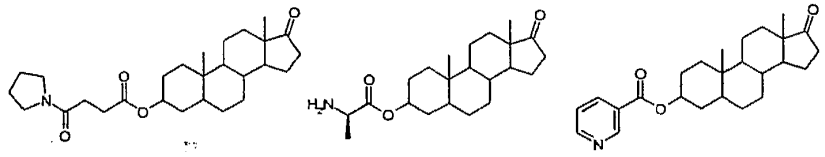
Samsung Bioepis Exhibit No. 1002
Page 8322



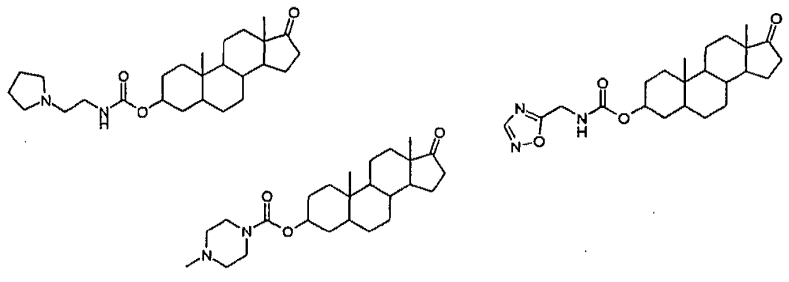
Examples of compounds of formula IA include,



R2 is preferably a diacid-derived or amino acid derived substituent, potentially including chloracetyl derivatives and acrylate derivatives, or optionally substituted aryls such as benzyl and heterobenzoyl. Examples of compounds of formula IB include,

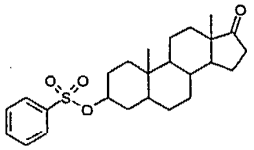
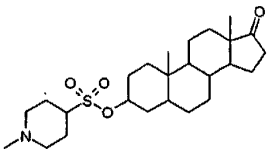


Examples of compounds of formula IC include,



Samsung Bioepis Exhibit No. 1002
Page 8323

R4 can be aromatic in nature and examples of suitable compounds of formula ID include,

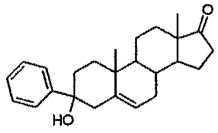
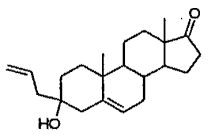
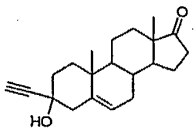



Other suitable analogs include the analogs wherein modifications have been performed at C-3 position by retaining OH or replacing OH with NH. These analogs are typically made by starting from C-17 acetal protected andrst-4-ene3, 17-dione, such as depicted in Figure 24. Compounds of Formula IE can be derived from Grignard reagents and possibly aryl-lithium reagents, such as aromatic in nature, and can also be alkynyl, alkenyl, and alkyl. Examples of R5 are

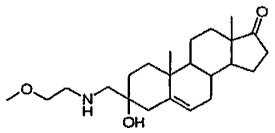
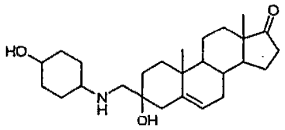
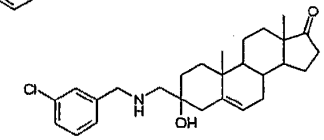
$CH_3(CH_2)_n$ where n=0 to 4



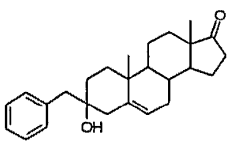
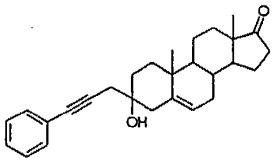
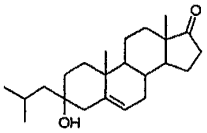

Examples of compounds of Formula IE include

R6 and R8 can independently be a diverse set of amines and can include amines possessing the functionalities as described for the R1 group. Examples of suitable compounds of Formula IF include,

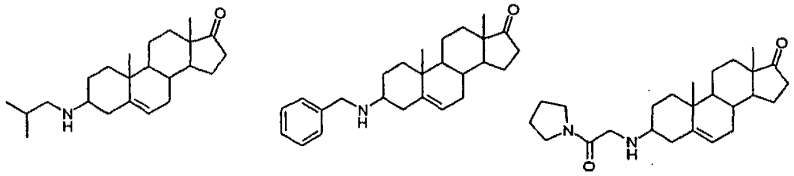




Suitable R7 groups can be derived from Grignard/organolithium reagents and so could encompass the functionalities described for R5. Examples of compounds of Formula IG include

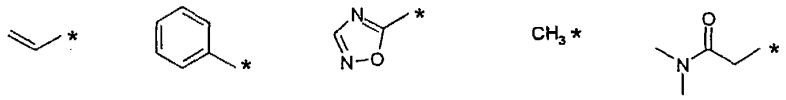




Examples of compounds of Formula IH include

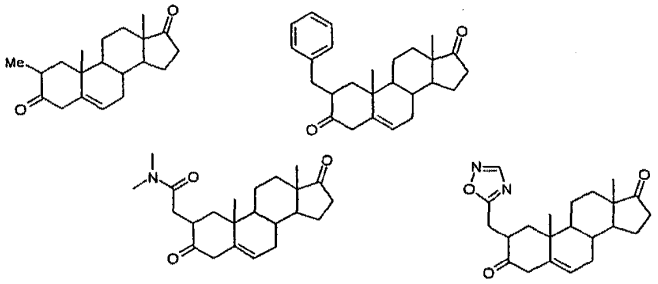
Samsung Bioepis Exhibit No. 1002
Page 8324



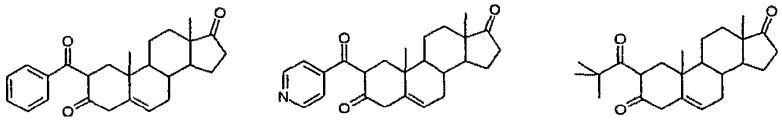
Other suitable analogs include those compounds wherein the C-2 position of DHEA was modified. Suitable modifications are depicted in Figure 25. R9 can be derived from alkylating agents, such as alkyl, benzyl, heterobenzyl, and derivatives of other activated halides. Examples of R9 include,



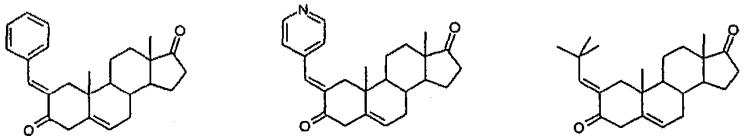
Example of Formula IJ compounds include,



R10 can be aromatic esters such as with aryl or heteroaryl ring or enolisable alkyl esters. Examples of compounds of formula IK include,

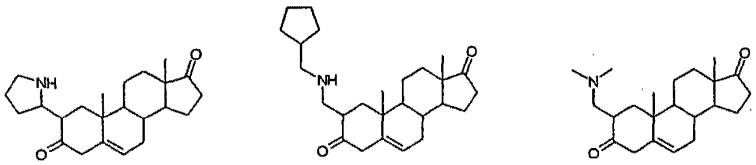


R11 can be a series of aromatic and heteroaromatic aldehydes such as benzene carboxaldehyde and substituted variants thereof, pyridine carboxaldehyde or non-enolisable aldehydes such as (CH3)3CCH=O. Examples of compounds of Formula IL include,



R12 can be a subset of an amine such as for R6. Examples of compounds of formula IN include,

Samsung Bioepis Exhibit No. 1002
Page 8325



Suitable modifications of the C-17 ketone of DHEA are depicted in Figure 26. The compounds depicted in Figure 23 can also be used as the first active agent.

Other suitable DHEA analogs are described in U.S. Patent 6,635,629; European Patent 934745; Dehydroepiandrosterone and analogs inhibit DNA binding of AP-1 and airway smooth muscle proliferation, Journal of Pharmacology and Experimental Therapeutics (1998), 285(2), 876-883; and Dehydroepiandrosterone and related steroids inhibit mitochondrial respiration in vitro, International Journal of Biochemistry (1989), 21(10), 1103-7, all of which are herein incorporated by reference in their entirety.

The second active agent is an anti-IgE antibody, or any functional fragment or antibody fragment thereof, capable of binding to IgE and preventing the IgE mediated allergic cascade. The anti-IgE antibody is capable of inhibiting any IgE-mediated disorder. Preferably, the anti-IgE antibody inhibits the IgE-mediated production of histamine. The range of anti-IgE antibody encompassed by this invention encompasses the compounds defined in U.S. Patent Nos. 5,543,144; 5,422,258; 5,653,980; 5,945,294; 5,965,709; and, 5,994,511 (the disclosure of which are incorporated herein by reference). Examples of the anti-IgE antibody are the antibody E-25 (or omalizumab), E-26, and E-27.

Preferably, the anti-IgE antibody is the anti-IgE antibody E-25 or omalizumab. More preferably, the anti-IgE antibody E-25 is modified by replacement of VL CDR-L1 residue 32Asp with Glu, along with the modification of VL CDR-L1 residues 27Gln, 28 Ser and 31 Tyr to Lys, Pro and Gly, respectively. Even more preferrably, the E-25 anti-IgE antibody has additional modifications at residues VH CDR2: 53Thr to Lys, 55Asp to Ser, 57Ser to Glu and 59Asn to Lys. Preferably, the anti-IgE antibody has an improved affinity to IgE. Preferably, the anti-IgE antibody comprises heavy and light chain residues comprising the sequence fragments labeled "e27" and "e26". Alternatively, the anti-IgE antibody comprises the full length heavy and light chain sequences labeled "E27" and "E26". Preferably, the anti-IgE antibody is capable of reducing or inhibiting the IgE-mediated production of histamine.

In one embodiment, the anti-IgE antibody comprises, or consists, the sequence "e25" of SEQ ID NOs:6-7. In another embodiment, the anti-IgE antibody comprises, or consists, the sequence "e26" of SEQ ID NOs:8-9. In another embodiment, the anti-IgE antibody comprises, or consists, the sequence "e27" of SEQ ID NOs:10-11. In another embodiment, the anti-IgE

Samsung Bioepis Exhibit No. 1002
Page 8326

antibody comprises an e26 sequence selected from the group consisting of: Fab fragment (SEQ ID NOs:12-13), sFv fragment (SEQ ID NO:15) and $F(ab')_2$ (SEQ ID NOs:17-18). In another embodiment, the anti-IgE antibody comprises an e27 sequence selected from the group consisting of Fab fragment (SEQ ID NOs:12-13), sFv fragment (SEQ ID NO:16) and $F(ab')_2$ (SEQ ID NOs:17 and 19).

The anti-IgE antibodies of this present invention can be prepared using the methods disclosed in U.S. Patent Nos. 5,543,144; 5,422,258; 5,653,980; 5,945,294; 5,965,709; and, 5,994,511 (the disclosure of which are incorporated herein by reference)..

Samsung Bioepis Exhibit No. 1002
Page 8327

The terms "protein" or "polypeptide" are intended to be used interchangeably. They refer to a chain of two (2) or more amino acids which are linked together with peptide or amide bonds, regardless of post-translational modification (e.g., glycosylation or phosphorylation). Antibodies are specifically intended to be within the scope of this definition. The polypeptides of this invention may comprise more than one subunit, where each subunit is encoded by a separate DNA sequence.

The phrase "substantially identical" with respect to an antibody polypeptide sequence shall be construed as an antibody exhibiting at least 70%, preferably 80%, more preferably 90% and most preferably 95% sequence identity to the reference polypeptide sequence. The term with respect to a nucleic acid sequence shall be construed as a sequence of nucleotides exhibiting at least about 85%, preferably 90% more preferably 95% and most preferably 97% sequence identity to the reference nucleic acid sequence. For polypeptides, the length of the comparison sequences will generally be at least 25 amino acids. For nucleic acids, the length will generally be at least 75 nucleotides.

The term "identity" or "homology" shall be construed to mean the percentage of amino acid residues in the candidate sequence that are identical with the residue of a corresponding sequence to which it is compared, after aligning the sequences and introducing gaps, if necessary to achieve the maximum percent identity for the entire sequence, and not considering any conservative substitutions as part of the sequence identity. Neither N- or C-terminal extensions nor insertions shall be construed as reducing identity or homology. Methods and computer programs for the alignment are well known in the art. Sequence identity may be measured using sequence analysis software (e.g., Sequence Analysis Software Package, Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Ave., Madison, Wis. 53705). This software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

The term "antibody" is used in the broadest sense, and specifically covers monoclonal antibodies (including full length monoclonal antibodies), polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies), and antibody fragments (e.g., Fab, $F(ab')_2$ and Fv) so long as they exhibit the desired biological activity. Antibodies (Abs) and immunoglobulins (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules which lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas.

Samsung Bioepis Exhibit No. 1002
Page 8328

Native antibodies and immunoglobulins are usually heterotetrameric glycoproteins of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies between the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VI H) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end. The constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain. Particular amino acid residues are believed to form an interface between the light and heavy chain variable domains (Clothia et al., *J. Mol. Biol.* 186, 651-66, 1985); Novotny et al., *Proc. Natl. Acad. Sci. USA* 82, 4592-4596 (1985) (the disclosure of which are incorporated herein by reference).

An "isolated" antibody is one which has been identified and separated and/or recovered from a component of the environment in which is was produced. Contaminant components of its production environment are materials which would interfere with diagnostic or therapeutic uses for the antibody, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the antibody will be purified as measurable by at least three different methods: 1) to greater than 95% by weight of antibody as determined by the Lowry method, and most preferably more than 99% by weight; 2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequentator; or 3) to homogeneity by SDS-PAGE under reducing or non-reducing conditions using Coomasie blue or, preferably, silver stain. Isolated antibody includes the antibody in situ within recombinant cells since at least one component of the antibody's natural environment will not be present. Ordinarily, however, isolated antibody will be prepared by at least one purification step.

A "species-dependent antibody," e.g., a mammalian anti-human IgE antibody, is an antibody which has a stronger binding affinity for an antigen from a first mammalian species than it has for a homologue of that antigen from a second mammalian species. Normally, the species-dependent antibody "bind specifically" to a human antigen (i.e., has a binding affinity (Kd) value of no more than about 1×10^{-7} M, preferably no more than about 1.times.10.sup.-8 and most preferably no more than about 1×10^{-9} M) but has a binding affinity for a homologue of the antigen from a second non-human mammalian species which is at least about 50 fold, or at least

30

Samsung Bioepis Exhibit No. 1002
Page 8329

about 500 fold, or at least about 1000 fold, weaker than its binding affinity for the human antigen. The species-dependent antibody can be of any of the various types of antibodies as defined above, but preferably is a humanized or human antibody.

The term "antibody mutant" refers to an amino acid sequence variant of an antibody wherein one or more of the amino acid residues have been modified. Such mutant necessarily have less than 100% sequence identity or similarity with the amino acid sequence having at least 75% amino acid sequence identity or similarity with the amino acid sequence of either the heavy or light chain variable domain of the antibody, more preferably at least 80%, more preferably at least 85%, more preferably at least 90%, and most preferably at least 95%. Since the method of the invention applies equally to both polypeptides, antibodies and fragments thereof, these terms are sometimes employed interchangeably.

The term "variable" in the context of variable domain of antibodies, refers to the fact that certain portions of the variable domains differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. However, the variability is not evenly distributed through the variable domains of antibodies. It is concentrated in three segments called complementarity determining regions (CDRs) also known as hypervariable regions both in the light chain and the heavy chain variable domains. There are at least two techniques for determining CDRs: (1) an approach based on cross-species sequence variability (i.e., Kabat et al., Sequences of Proteins of Immunological Interest (National Institute of Health, Bethesda, Md. 1987); and (2) an approach based on crystallographic studies of antigen-antibody complexes (Chothia, C. et al. (1989), Nature 342: 877). With respect to Applicants' anti-IgE antibody, certain CDRs were defined by combining the Kabat et al. and Chothia et al. approaches. The more highly conserved portions of variable domains are called the framework (FR). The variable domains of native heavy and light chains each comprise four FR regions, largely a adopting a .beta.-sheet configuration, connected by three CDRs, which form loops connecting, and in some cases forming part of, the .beta.-sheet structure. The CDRs in each chain are held together in close proximity by the FR regions and, with the CDRs from the other chain, contribute to the formation of the antigen binding site of antibodies (see Kabat et al.) The constant domains are not involved directly in binding an antibody to an antigen, but exhibit various effector function, such as participation of the antibody in antibody-dependent cellular toxicity.

The term "antibody fragment" refers to a portion of a full-length antibody, generally the antigen binding or variable region. Examples of antibody fragments include Fab, Fab', $F(ab')_2$ and

Samsung Bioepis Exhibit No. 1002
Page 8330

Fv fragments. Papain digestion of antibodies produces two identical antigen binding fragments, called the Fab fragment, each with a single antigen binding site, and a residual "Fc" fragment, so-called for its ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen binding fragments which are capable of cross-linking antigen, and a residual other fragment (which is termed pFc'). Additional fragments can include diabodies, linear antibodies, single-chain antibody molecules, and multispecific antibodies formed from antibody fragments. As used herein, "functional fragment" with respect to antibodies, refers to Fv, F(ab) and $F(ab')_2$ fragments.

An "Fv" fragment is the minimum antibody fragment which contains a complete antigen recognition and binding site. This region consists of a dimer of one heavy and one light chain variable domain in a tight, non-covalent association (V_H -V_L dimer). It is in this configuration that the three CDRs of each variable domain interact to define an antigen binding site on the surface of the V_H -V_L dimer. Collectively, the six CDRs confer antigen binding specificity to the antibody. However, even a single variable domain (or half of an Fv comprising only three CDRs specific for an antigen) has the ability to recognize and bind antigen, although at a lower affinity than the entire binding site.

The Fab fragment (also designated as F(ab)) also contains the constant domain of the light chain and the first constant domain (CH1) of the heavy chain. Fab' fragments differ from Fab fragments by the addition of a few residues at the carboxyl terminus of the heavy chain CH1 domain including one or more cysteines from the antibody hinge region. Fab'-SH is the designation herein for Fab' in which the cysteine residue(s) of the constant domains have a free thiol group. F(ab') fragments are produced by cleavage of the disulfide bond at the hinge cysteines of the $F(ab')_2$ pepsin digestion product. Additional chemical couplings of antibody fragments are known to those of ordinary skill in the art.

The light chains of antibodies (immunoglobulin) from any vertebrate species can be assigned to one of two clearly distinct types, called kappa (κ) and lambda (λ), based on the amino sequences of their constant domain.

The term "monoclonal antibody" as used herein refers to an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site. Furthermore, in contrast to conventional (polyclonal) antibody preparations which typically include different antibodies directed against different determinants (epitopes), each

Samsung Bioepis Exhibit No. 1002
Page 8331

monoclonal antibody is directed against a single determinant on the antigen. In additional to their specificity, the monoclonal antibodies are advantageous in that they are synthesized by the hybridoma culture, uncontaminated by other immunoglobulins. The modifier "monoclonal" indicates the character of the antibody indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler et al., *Nature* 256, 495 (1975), or may be made by recombinant methods, e.g., as described in U.S. Patent No. 4,816,567. The monoclonal antibodies for use with the present invention may also be isolated from phage antibody libraries using the techniques described in Clackson et al. *Nature* 352: 624-628 (1991), as well as in Marks et al., *J. Mol. Biol.* 222: 581-597 (1991). (The disclosure of these references and patent are incorporated herein by reference.)

The monoclonal antibodies herein specifically include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies, so long as they exhibit the desired biological activity (U.S. Pat. No. 4,816,567); Morrison et al. Proc. Natl. Acad. Sci. 81, 6851-6855 (1984).

"Humanized" forms of non-human (e.g. murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', $F(ab')_2$ or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and corresponding non-human residues. Furthermore, humanized antibody may comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. These modifications are made to further refine and optimize antibody performance. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all

Samsung Bioepis Exhibit No. 1002
Page 8332

or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin. For further details, see: Jones et al., *Nature* 321, 522-525 (1986); Reichmann et al., *Nature* 332, 323-329 (1988) and Presta, *Curr. Op. Struct. Biol.* 2, 593-596 (1992) (the disclosure of which are incorporated herein by reference.).

"Single-chain Fv" or "sFv" antibody fragments comprise the VH and VL domains of an antibody, wherein these domains are present in a single polypeptide chain. Generally, the Fv polypeptide further comprises a polypeptide linker between the VH and VL domains which enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg et al. eds. Springer-Verlag, New York, pp. 269-315 (1994).

The term "diabodies" refers to a small antibody fragments with two antigen-binding sites, which fragments comprise a heavy chain variable domain (VH) connected to a light chain variable domain (VL) in the same polypeptide chain (VH-VL). By using a linker that is too short to allow pairing between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161, and Hollinger et al., Proc. Natl. Acad. Sci. USA 90: 6444-6448 (1993) (the disclosure of these references and patents are incorporated herein by reference.).

The phrase "functional fragment or analog" of an antibody is a compound having qualitative biological activity in common with a full-length antibody. For example, a functional fragment or analog of an anti-IgE antibody is one which can bind to an IgE immunoglobulin in such a manner so as to prevent or substantially reduce the ability of such molecule from having the ability to bind to the high affinity receptor, FcεRI.

The term "amino acid" and "amino acids" refer to all naturally occuring L-α-amino acids. The amino acids are identified as hereinafter described under section A. Antibody Preparation: (iv) Generation of mutant antibodies. The term "amino acid variant" refers to molecules with some differences in their amino acid sequences as compared to a native amino acid sequence.

"Substitutional" variants are those that have at least one amino acid residue in a native sequence removed and a different amino acid inserted in its place at the same position. The substitutions may be single, where only one amino acid in the molecule as been substituted, or they may be multiple, where two or more amino acids have been substituted in the same

Samsung Bioepis Exhibit No. 1002
Page 8333

molecule. "Insertional" variants are those with one or more amino acids inserted immediately adjacent to an amino acid at a particular position in a native sequence. Immediately adjacent to an amino acid means connected to either the α-carboxyl or α-amino functional group of the amino acid. "Deletional" variants are those with one or more amino acids in the native amino acid sequence removed. Ordinarily, deletional variants will have one or two amino acids deleted in a particular region of the molecule.

As used herein, anti-human IgE antibody means an antibody which binds to human IgE in such a manner so as to inhibit or substantially reduce the binding of such IgE to the high affinity receptor, FcεRI. Preferably this anti-IgE antibody is E-25.

As used herein, the term "IgE-mediated disorder" means a condition or disease which is characterized by the overproduction and/or hypersensitivity to the immunoglobulin IgE. Specifically it should be construed to include conditions associated with anaphylactic hypersensitivity and atopic allergies, including for example: asthma, allergic rhinitis and conjunctivitis (hay fever), eczema, urticaria and food allergies.

Preferably, the anti-IgE antibody is capable of reducing or preventing any IgE-mediated disorder. The anti-IgE antibody can be substantially identical to the anti-IgE antibody E-25 and capable of binding IgE and is capable of reducing or preventing any IgE-mediated disorder. The anti-IgE antibody can be a mutant of an anti-IgE antibody, such as E-25, and capable of binding IgE and is capable of reducing or preventing any IgE-mediated disorder. The mutant can comprise a substitutional, insertional and/or deletional variant. Preferably, the anti-IgE antibody is humanized. Preferably, the anti-IgE antibody is isolated. The anti-IgE antibody can be a polyclonal antibody or monoclonal antibody. The anti-IgE antibody can be a functional fragment or analog of a anti-IgE antibody.

A preferred second active agent is omalizumab, which is a recombinant DNA-derived humanized IgG1κ monoclonal antibody that selectively binds to human IgE (Xolair®). The antibody has a molecular weight of approximately 149 kilodaltons. Xolair is produced by a Chinese hamster ovary cell suspension culture in a nutrient medium containing the antibiotic gentamicin. Gentamicin is not detectable in the final product.

Omalizumab is a sterile, white, preservative-free, lyophilized powder contained in a single-use vial that is reconstituted with Sterile Water for Injection (SWFI), USP, and administered as a subcutaneous (SC) injection. A vial of Xolair® contains 202.5 mg of omalizumab, 145.5 mg sucrose, 2.8 mg L-histidine hydrochloride monohydrate, 1.8 mg L-

Samsung Bioepis Exhibit No. 1002
Page 8334

histidine, and 0.5 mg polysorbate 20, and is designed to deliver 150 mg of omalizumab in 1.2 mL after reconstitution with 1.4 mL SWFI, USP.

Omalizumab inhibits the binding of IgE to the high-affinity IgE receptor (FcεRI) on the surface of mast cells and basophils. Reduction in surface-bound IgE on FcεRI-bearing cells limits the degree of release of mediators of the allergic response. Treatment with omalizumab also reduces the number of FcεRI receptors on basophils in atopic patients.

Xolair® is indicated for adults and adolescents (12 years of age and above) with moderate to severe persistent asthma who have a positive skin test or *in vitro* reactivity to a perennial aeroallergen and whose symptoms are inadequately controlled with inhaled corticosteroids. Omalizumab has been shown to decrease the incidence of asthma exacerbations in these patients. The preferred dosage for xolair (omalizumab) is 150 to 375 mg administered subcutaneously every 2 or 4 weeks.

Asthma is characterized by a complex inflammatory response to antigens. Soluble mediators and inflammatory cells contribute to the symptoms and the airway obstruction in asthma. Medications from different therapeutic classes have been developed to antagonize specific parts of the inflammatory cascade. IgE is an immunoglobulin produced after exposure to allergens that have two forms, soluble and bound. IgE is very specific to a given allergen. It is pre-formed and therefore immediately available to bind to its receptor on primed effector cells, such as basophils and mast cells. Formation of a complex of many bound IgE-IgE receptors ('capping') on an effector cell is a potent stimulus for release of many pro-inflammatory mediators (histamine, leukotrienes, bradykinin, platelet activating factor etc.) The remarkable attributes of the IgE immune response is its immediacy and its specificity. Within seconds to minutes after exposure to a unique allergen, mast cells and basophils will degranulate, releasing their mediators and causing prompt itching, sneezing, swelling, bronchospasm, cough and mucus secretion. One can document this series of events by monitoring the degree of airway obstruction with spirometry. The typical response after exposure to allergen is a biphasic response with severe reductions in airflow occurring within minutes after exposure (early asthmatic response) that recovers to near normal by 1-2 hours and a late asthmatic response that occurs from six to 24 hours later. The early asthmatic response is believed to be caused by the immediate release of mediators such as those triggered by IgE. The early asthmatic response is most amenable to bronchodilators and to therapy with anti-IgE antibody. The later asthmatic response is due to inflammatory cellular influx and is amenable to therapy with glucocorticoid therapy. Fahy et al (Am J Respir Crit Care Med, 1997;155:1828) have shown that anti-IgE antibody therapy is

Samsung Bioepis Exhibit No. 1002
Page 8335

effective in reducing both the early (by 60%) and the late phase asthmatic response by approximately 50%. We have shown that DHEA-S is effective in reducing the late asthmatic response by 33% in patients after five days of dosing. Not intending to be limited to one mechanism of action, the combination of DHEA-S with anti-IgE therapy would have an additive effect on the late asthmatic response because both compounds affect different pathways of the allergic response. In addition to DHEA-S, other suitable non-glucocorticoid steroids can be used as the first active agent, including, but not limited to, epiandrosterone and derivative, analogs, and pharmaceutically acceptable salts thereof. For example, such as the compounds depicted by Formulas I, III, and IV, herein.

The first and second active agents are used to treat respiratory and lung diseases, and any of the additional agents listed below, may be administered per se or in the form of pharmaceutically acceptable salts, as discussed above, all being referred to as "active compounds or agents". The first and second active agents may also be administered in combination with one another, in the form of separate, or jointly in, pharmaceutically or veterinarily acceptable formulation(s). The active compounds or their salts may be administered either systemically or topically, as discussed below.

The present invention also provides for methods for treating asthma, COPD, or other respiratory diseases comprising administering the composition to a subject in need of such treatment. The method is for prophylactic or therapeutic purposes. The method comprises an in vivo method. The method is effective for treating a plurality of diseases, whatever their cause, including steroid administration, abnormalities in adenosine or adenosine receptor metabolism or synthesis, or any other cause. The method comprises treating respiratory and lung diseases, whether by reducing adenosine or adenosine receptor levels, reducing hypersensitivity to adenosine, or any other mechanism, particularly in the lung, liver, heart and brain, or any organ that is need of such treatment. Other respiratory diseases referred to herein include cystic fibrosis (CF), dyspnea, emphysema, wheezing, pulmonary hypertension, pulmonary fibrosis, lung cancer, hyper-responsive airways, increased adenosine or adenosine receptor levels, particularly those associated with infectious diseases, pulmonary bronchoconstriction, lung inflammation, lung allergies, surfactant depletion, chronic bronchitis, bronchoconstriction, difficult breathing, impeded and obstructed lung airways, adenosine test for cardiac function, pulmonary vasoconstriction, impeded respiration, Acute Respiratory Distress Syndrome (ARDS), administration of certain drugs, such as adenosine and adenosine level increasing drugs, and other drugs for, e.g. treating SupraVentricular Tachycardia (SVT), and the administration of adenosine

Samsung Bioepis Exhibit No. 1002
Page 8336

stress tests, infantile Respiratory Distress Syndrome (infantile RDS), pain, allergic rhinitis, decreased lung surfactant, severe acute respiratory syndrome (SARS), among others.

In one embodiment, the invention is a method for the prophylaxis or treatment of asthma comprising administering the composition to a subject in need of such treatment an amount of the composition sufficient for the prophylaxis or treatment of asthma in the subject.

In one embodiment, the invention is a method for the prophylaxis or treatment of COPD comprising administering the composition to a subject in need of such treatment an amount of the composition sufficient for the prophylaxis or treatment of COPD in the subject.

In one embodiment, the invention is a method for the prophylaxis or treatment of bronchoconstriction, lung inflammation or lung allergy comprising administering the composition to a subject in need of such treatment an amount of the composition sufficient for the prophylaxis or treatment of bronchoconstriction, lung inflammation or lung allergy in the subject.

In one embodiment, the invention is a method for the reducing or depleting adenosine in a subject's tissue comprising administering the composition to a subject in need of such treatment an amount of the composition sufficient to reduce or deplete adenosine in the subject's tissue.

The present invention also provides for a use of the first active agent and the second active agent in the manufacture of a medicament for the treatment of asthma, COPD, or other respiratory diseases, including lung cancer. The medicament comprises the composition described throughout this disclosure.

The daily dosage of the first active agent and the second active agent to be administered to a subject will vary with the overall treatment programmed, the first active agent and the second active agent to be employed, the type of formulation, the route of administration and the state of the patient. Examples 11 to 14 show aerosolized preparations in accordance with the invention for delivery with a device for respiratory or nasal administration, or administration by inhalation. For intrapulmonary administration, liquid preparations are preferred. In the case of other bioactive agents, there exist FDA recommended amounts for supplementing a person's dietary intake with additional bioactive agents, such as in the case of vitamins and minerals. However, where employed for the treatment of specific conditions or for improving the immune response of a subject they may be utilized in dosages hundreds and thousands of times higher. Mostly, the pharmacopeia's recommendations cover a very broad range of dosages, from which the medical artisan may draw guidance. Amounts for the exemplary agents described in this patent may be in the range of those currently being recommended for daily consumption, below or above those levels. The treatment may typically begin with a low dose of a bronchodilator in combination

Samsung Bioepis Exhibit No. 1002
Page 8337

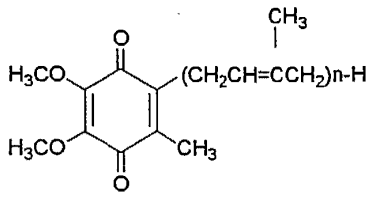
with a non-glucocorticoid steroid, or other bioactive agents as appropriate, and then a titration up of the dosage for each patient. Higher and smaller amounts, including initial amounts, however, may be administered within the confines of this invention as well.

Preferable ranges for the first and second active agents, or any other therapeutic agent, employed here will vary depending on the route of administration and type of formulation employed, as an artisan will appreciate and manufacture in accordance with known procedures and components. The active compounds may be administered as one dose (once a day) or in several doses (several times a day). The compositions and method of preventing and treating respiratory, cardiac, and cardiovascular diseases may be used to treat adults and infants, as well as non-human animals afflicted with the described conditions. Although the present invention is concerned primarily with the treatment of human subjects, it may also be employed, for veterinary purposes in the treatment of non-human mammalian subjects, such as dogs and cats as well as for large domestic and wild animals. The terms "high" and "low" levels of "adenosine" and "adenosine receptors" as well as "adenosine depletion" are intended to encompass both, conditions where adenosine levels are higher than, or lower (even depleted) when compared to previous adenosine levels in the same subject, and conditions where adenosine levels are within the normal range but, because of some other condition or alteration in that patient, a therapeutic benefit would be achieved in the patient by decreasing or increasing adenosine or adenosine receptor levels or hypersensitivity. Thus, this treatment helps regulate (titrate) the patient in a custom tailored manner. Whereas the administration of the first active agent may decrease or even deplete adenosine levels in a subject having either normal or high levels prior to treatment, the further administration of the second active agent will improve the subject's respiration in a short period of time. The further addition of other therapeutic agents will help titrate undesirably low levels of adenosine, which may be observed upon the administration of the present treatment, particularly until an optimal titration of the appropriate dosages is attained.

Other therapeutic agents that may be incorporated into the present composition are one or more of a variety of therapeutic agents that are administered to humans and animals.

The composition can further comprise, in addition to the first and second active agents, a ubiquinone and/or folinic acid. A ubiquinone is a compound represented by the formula:

Samsung Bioepis Exhibit No. 1002
Page 8338



(II)

or pharmaceutically acceptable salt thereof.

Preferably, the ubiquinone is a compound according to the chemical formula given above, wherein n=1-10 (Coenzymes Q_{1-10}), more preferably n=6-10, (Coenzymes Q_{6-10}) and most preferably n=10 (Coenzyme Q_{10}). The ubiquinone is administered in a therapeutic amount for treating the targeted disease or condition, and the dosage will vary depending upon the condition of the subject, other agents being administered, the type of formulation employed, and the route of administration. The ubiquinone is preferably administered in a total amount per day of about 0.1, about 1, about 3, about 5, about 10, about 15, about 30 to about 50, about 100, about 150, about 300, about 600, about 900, about 1200 mg/kg body weight. More preferred the total amount per day is about 1 to about 150 mg/kg, about 30 to about 100 mg/kg, and most preferred about 5 to about 50 mg/kg. Ubiquinone is a naturally occurring substance and is available commercially.

The active agents of this invention are provided within broad amounts of the composition. For example, the active agents may be contained in the composition in amounts of about 0.001%, about 1%, about 2%, about 5%, about 10%, about 20%, about 40%, about 90%, about 98%, about 99.999% of the composition. The amount of each active agent may be adjusted when, and if, additional agents with overlapping activities are included as discussed in this patent. The dosage of the active compounds, however, may vary depending on age, weight, and condition of the subject. Treatment may be initiated with a small dosage, e.g. less than the optimal dose, of the first active agent of the invention. This may be similarly done with the second active agent, until a desirable level is attained. Or vice versa, for example in the case of multivitamins and/or minerals, the subject may be stabilized at a desired level of these products and then administered the first active compound. The dose may be increased until a desired and/or optimal effect under the circumstances is reached. In general, the active agent is preferably administered at a concentration that will afford effective results without causing any unduly harmful or deleterious side effects, and may be administered either as a single unit dose, or if desired in convenient subunits administered at suitable times throughout the day. The second therapeutic or diagnostic agent(s) is (are) administered in amounts which are known in the art to be effective for the intended application. In cases where the second agent has an overlapping

Samsung Bioepis Exhibit No. 1002
Page 8339

activity with the principal agent, the dose of one of the other or of both agents may be adjusted to attain a desirable effect without exceeding a dose range that avoids untoward side effects. Thus, for example, when other analgesic and anti-inflammatory agents are added to the composition, they may be added in amounts known in the art for their intended application or in doses somewhat lower that when administered by themselves.

Pharmaceutically acceptable salts should be pharmacologically and pharmaceutically or veterinarily acceptable, and may be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts. Organic salts and esters are also suitable for use with this invention. The active compounds are preferably administered to the subject as a pharmaceutical or veterinary composition, which includes systemic and topical formulations. Among these, preferred are formulations suitable for inhalation, or for respirable, buccal, oral, rectal, vaginal, nasal, intrapulmonary, ophthalmic, optical, intracavitary, intratraccheal, intraorgan, topical (including buccal, sublingual, dermal and intraocular), parenteral (including subcutaneous, intradermal, intramuscular, intravenous and intraarticular) and transdermal administration, among others.

The present invention also provides for a kit comprising the composition and a delivery device. The compositions may conveniently be presented in single or multiple unit dosage forms as well as in bulk, and may be prepared by any of the methods which are well known in the art of pharmacy. The composition, found in the kit, whether already formulated together or where the first and second active agents are separately provided along with other ingredients, and instructions for its formulation and administration regime. The kit may also contain other agents, such as those described in this patent and, for example, when for parenteral administration, they may be provided with a carrier in a separate container, where the carrier may be sterile. The present composition may also be provided in lyophilized form, and in a separate container, which may be sterile, for addition of a liquid carrier prior to administration. See, e.g. U.S. Patent No. 4,956,355; UK Patent No. 2,240,472; EPO Patent Application Serial No. 429,187; PCT Patent Publication WO 91/04030; Mortensen, S. A., et al., *Int. J. Tiss. Reac.* XII(3): 155-162 (1990); Greenberg, S. et al., *J. Clin. Pharm.* 30: 596-608 (1990); Folkers, K., et al., *Proc. Natl. Acad. Sci. USA* 87: 8931-8934 (1990), the relevant preparatory and compound portions of which are incorporated by reference above.

The present composition is provided in a variety of systemic and topical formulations. The systemic or topical formulations of the invention are selected from the group consisting of oral, intrabuccal, intrapulmonary, rectal, intrauterine, intradermal, topical, dermal, parenteral,

Samsung Bioepis Exhibit No. 1002
Page 8340

intratumor, intracranial, intrapulmonary, buccal, sublingual, nasal, subcutaneous, intravascular, intrathecal, inhalable, respirable, intraarticular, intracavitary, implantable, transdermal, iontophoretic, intraocular, ophthalmic, vaginal, optical, intravenous, intramuscular, intraglandular, intraorgan, intralymphatic, slow release and enteric coating formulations. The actual preparation and compounding of these different formulations is known in the art and need not be detailed here. The composition may be administered once or several times a day.

Formulations suitable for respiratory, nasal, intrapulmonary, and inhalation administration are preferred, as are topical, oral and parenteral formulations. All methods of preparation include the step of bringing the active compound into association with a carrier which constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product into desired formulations.

Compositions suitable for oral administration may be presented in discrete units, such as capsules, cachets, lozenges, or tablets, each containing a predetermined amount of the active compound; as a powder or granules; as a solution or a suspension in an aqueous or non-aqueous liquid; or as an oil-in-water or water-in-oil emulsion.

Compositions suitable for parenteral administration comprise sterile aqueous and non-aqueous injection solutions of the active compound, which preparations are preferably isotonic with the blood of the intended recipient. These preparations may contain anti-oxidants, buffers, bacteriostats and solutes which render the compositions isotonic with the blood of the intended recipient. Aqueous and non-aqueous sterile suspensions may include suspending agents and thickening agents. The compositions may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried or lyophilized condition requiring only the addition of the sterile liquid carrier, for example, saline or water-for-injection immediately prior to use.

Nasal and instillable formulations comprise purified aqueous solutions of the active compound with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes.

Formulations for rectal or vaginal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids.

Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye. Otical formulations

Samsung Bioepis Exhibit No. 1002
Page 8341

are generally prepared in viscous carriers, such as oils and the like, as is known in the art, so that they may be easily administered into the ear without spilling.

Compositions suitable for topical application to the skin preferably take the form of an ointment, cream, lotion, paste, gel, spray, aerosol, or oil. Carriers which may be used include Vaseline, lanolin, polyethylene glycols, alcohols, transdermal enhancers, and combinations of two or more thereof. Compositions suitable for transdermal administration may be presented as discrete patches adapted to remain in intimate contact with the epidermis of the recipient for a prolonged period of time.

The first and second active agents disclosed herein may be administered into the respiratory system either by inhalation, respiration, nasal administration or intrapulmonary instillation (into the lungs) of a subject by any suitable means, and are preferably administered by generating an aerosol or spray comprised of powdered or liquid nasal, intrapulmonary, respirable or inhalable particles. The respirable or inhalable particles comprising the active compound are inhaled by the subject, i.e, by inhalation or by nasal administration or by instillation into the respiratory tract or the lung itself. The formulation may comprise respirable or inhalable liquid or solid particles of the active compound that, in accordance with the present invention, include respirable or inhalable particles of a size sufficiently small to pass through the mouth and larynx upon inhalation and continue into the bronchi and alveoli of the lungs. In general, particles ranging from about 0.05, about 0.1, about 0.5, about 1, about 2 to about 4, about 6, about 8, about 10 μm in diameter. More particularly, about 0.5 to less than about 5 μm in diameter, are respirable or inhalable. Particles of non-respirable size which are included in an aerosol or spray tend to deposit in the throat and be swallowed. The quantity of non-respirable particles in the aerosol is, thus, preferably minimized. For nasal administration or intrapulmonary instillation, a particle size in the range of about 8, about 10, about 20, about 25 to about 35, about 50, about 100, about 150, about 250, about 500 μm (diameter) is preferred to ensure retention in the nasal cavity or for instillation and direct deposition into the lung. Liquid formulations may be squirted into the respiratory tract (nose) and the lung, particularly when administered to newborns and infants.

Liquid pharmaceutical compositions of active compound for producing an aerosol may be prepared by combining the active compound with a stable vehicle, such as sterile pyrogen free water. Solid particulate compositions containing respirable dry particles of micronized active compound may be prepared by grinding dry active compound with a mortar and pestle, and then passing the micronized composition through a 400 mesh screen to break up or separate out large

Samsung Bioepis Exhibit No. 1002
Page 8342

agglomerates. A solid particulate composition comprised of the active compound may optionally contain a dispersant that serves to facilitate the formation of an aerosol. A suitable dispersant is lactose, which may be blended with the active compound in any suitable ratio, e.g., a 1 to 1 ratio by weight. The U.S. Patent Application Ser. Nos. 10/462,901 and 10/462,927 disclose a stable dry powder formulation of DHEA in a nebulizable form and a stable dry powder formulation of dihydrate crystal form of DHEA-S, respectively (these patent applications are herein incorporated by reference in their entirety).

Aerosols of liquid particles comprising the active compound may be produced by any suitable means, such as with a nebulizer. See, e.g. U.S. Patent No. 4,501,729 (the disclosure of which is incorporated herein by reference). Nebulizers are commercially available devices which transform solutions or suspensions of the active ingredient into a therapeutic aerosol mist either by means of acceleration of a compressed gas, typically air or oxygen, through a narrow venturi orifice or by means of ultrasonic agitation. Suitable compositions for use in nebulizer consist of the active ingredient in liquid carrier, the active ingredient comprising up to 40% w/w composition, but preferably less than 20% w/w carrier being typically water or a dilute aqueous alcoholic solution, preferably made isotonic with body fluids by the addition of, for example sodium chloride. Optional additives include preservatives if the composition is not prepared sterile, for example, methyl hydroxybenzoate, anti-oxidants, flavoring agents, volatile oils, buffering agents and surfactants. Aerosols of solid particles comprising the active compound may likewise be produced with any sold particulate medicament aerosol generator. Aerosol generators for administering solid particulate medicaments to a subject product particles which are respirable, as explained above, and generate a volume of aerosol containing a predetermined metered dose of a medicament at a rate suitable for human administration. Examples of such aerosol generators include metered dose inhalers and insufflators.

The composition may be delivered with any delivery device that generates liquid or solid particulate aerosols, such as aerosol or spray generators. These devices produce respirable particles, as explained above, and generate a volume of aerosol or spray containing a predetermined metered dose of a medicament at a rate suitable for human or animal administration. One illustrative type of solid particulate aerosol or spray generator is an insufflator, which are suitable for administration of finely comminuted powders. In the insufflator, the powder, e.g. a metered dose of the composition effective to carry out the treatments described herein, is contained in a capsule or a cartridge. These capsules or cartridges are typically made of gelatin, foil or plastic, and may be pierced or opened in situ, and the powder

Samsung Bioepis Exhibit No. 1002
Page 8343

delivered by air drawn through the device upon inhalation or by means of a manually-operated pump. The composition employed in the insufflator may consist either solely of the first and second agents or of a powder blend comprising the first and second agents, typically comprising from 0.01 to 100 % w/w of the composition. The composition generally contains the first and second agents in an amount of about 0.01% w/w, about 1% w/w/, about 5% w/w, to about 20%, w/w, about 40% w/w, about 99.99% w/w. Other ingredients, and other amounts of the agent, however, are also suitable within the confines of this invention.

In one embodiment, the composition is delivered by a nebulizer. This means is especially useful for patients or subjects who are unable to inhale or respire the composition under their own efforts. In serious cases, the patients or subjects are kept alive through artificial respirator. The nebulizer can use any pharmaceutically or veterinarily acceptable carrier, such as a weak saline solution. The nebulizer is the means by which the powder pharmaceutical composition is delivered to the target of the patients or subjects in the airways.

The composition is also provided in various forms that are tailored for different methods of administration and routes of delivery. In one embodiment, the composition comprises a respirable formulation, such as an aerosol or spray. The composition of the invention is provided in bulk, and in unit form, as well as in the form of an implant, a capsule, blister or cartridge, which may be openable or piercable as is known in the art. A kit is also provided, that comprises a delivery device, and in separate containers, the composition of the invention, and optionally other excipient and therapeutic agents, and instructions for the use of the kit components.

In one embodiment, the composition is delivered using suspension metered dose inhalation (MDI) formulation. Such a MDI formulation can be delivered using a delivery device using a propellant such as hydrofluroalkane (HFA). Preferably, the HFA propellants contain 100 parts per million (PPM) or less of water.

In one embodiment, the delivery device comprises a dry powder inhalator (DPI) that delivers single or multiple doses of the composition. The single dose inhalator may be provided as a disposable kit which is sterilely preloaded with enough formulation for one application. The inhalator may be provided as a pressurized inhalator, and the formulation in a piercable or openable capsule or cartridge. The kit may optionally also comprise in a separate container an agent such as other therapeutic compounds, excipients, surfactants (intended as therapeutic agents as well as formulation ingredients), antioxidants, flavoring and coloring agents, fillers, volatile oils, buffering agents, dispersants, surfactants, antioxidants, flavoring agents, bulking

Samsung Bioepis Exhibit No. 1002
Page 8344

agents, propellants and preservatives, among other suitable additives for the different formulations.

Having now generally described this invention, the same will be better understood by reference to certain specific examples, which are included herein for purposes of illustration only and are not intended to be limiting of the invention or any embodiment thereof, unless so specified.

EXAMPLES

Examples 1 and 2: In vivo Effects of Folinic Acid and DHEA on Adenosine Levels

Young adult male Fischer 344 rats (120 grams) were administered dehydroepiandrosterone (DHEA) (300 mg/kg) or methyltestosterone (40 mg/kg) in carboxymethylcellulose by gavage once daily for fourteen days. Folinic acid (50 mg/kg) was administered intraperitoneally once daily for fourteen days. On the fifteenth day, the animals were sacrificed by microwave pulse (1.33 kilowatts, 2450 megahertz, 6.5 seconds (s)) to the cranium, which instantly denatures all brain protein and prevents further metabolism of adenosine. Hearts were removed from animals and flash frozen in liquid nitrogen with 10 s of death. Liver and lungs were removed en bloc and flash frozen with 30 s of death. Brain tissue was subsequently dissected. Tissue adenosine was extracted, derivatized to 1, N6-ethenoadenosine and analyzed by high performance liquid chromatography (HPLC) using spectrofluorometric detection according to the method of Clark and Dar (J. of Neuroscience Methods 25:243 (1988)). Results of these experiments are summarized in Table 1 below. Results are expressed as the mean ±SEM, with κ $p<0.05$ compared to control group and ψ $p<0.05$ compared to DHEA or methyltestosterone-treated groups.

Table 1: In vivo Effect of DHEA, δ -1-methyltestosterone and Folinic Acid on Adenosine Levels in various Rat Tissues

	Intracellular adenosine (nmols)/mg protein			
Treatment	Heart	Liver	Lung	Brain
Control	10.6 ± 0.6 (n=12) κ	14.5 ± 1.0 (n=12) κ	3.1 ± 0.2 (n=6) κ	0.5 ± 0.04 (n=12) κ
DHEA (300 mg/kg)	6.7 ± 0.5 (n=12) κ	16.4 ± 1.4 (n=12) κ	2.3 ± 0.3 (n=6) κ	0.19 ± 0.01 (n=12) κ
Methyltestosterone (40 mg/kg)	8.3 ± 1.0 (n=6) κ	16.5 ± 0.9 (n=6) κ	N.D.	0.42 ± 0.06 (n=6) κ

Samsung Bioepis Exhibit No. 1002
Page 8345

Methyltestosterone (120 mg/kg)	6.0 ± 0.4 (n=6) κ	5.1 ± 0.5 (n=6) κ	N.D.	0.32 ± 0.03 (n=6) κ
Folinic Acid (50 mg/kg)	12.4 ± 2.1 (n=5) κ	16.4 ±2.4 (n=5) κ	N.D.	0.72 ± 0.09 (n=5) κ
DHEA (300 mg/kg) + Folinic Acid (50 mg/kg)	11.1 ± 0.6 (n=5) ψ	18.8 ± 1.5 (n=5) ψ	N.D.	0.55 ± 0.09 (n=5) ψ
Methyltestosterone (120 mg/kg) + Folinic Acid (50 mg/kg)	9.1 ± 0.4 (n=6) ψ	N.D.	N.D.	0.60 ± 0.06 (n=6) ψ

N.D.=Not determined

The results of these experiments indicate that rats administered DHEA or methyltestosterone daily for two weeks showed multi-organ depletion of adenosine. Depletion was dramatic in brain (60% depletion for DHEA, 34% for high dose methyltestosterone) and heart (37% depletion for DHEA, 22% depletion for high dose methyltestosterone). Coadministration of folinic acid completely abrogated steroid-mediated adenosine depletion. Folinic acid administered alone induce increase in adenosine levels for all organs studied.

Example 3: Airjet Milling of Anhydrous DHEA-S and Determination of Respirable Dose

DHEA-S is evaluated as an asthma therapy. The solid-state stability of sodium dehydroepiandrostenone sulfate (NaDHEA-S) has been studied for both bulk and milled material (Nakagawa, H., Yoshiteru, T., and Fujimoto, Y. (1981) *Chem. Pharm. Bull.* 29(5) 1466-1469; Nakagawa, H., Yoshiteru, T., and Sugimoto, I. (1982) *Chem. Pharm. Bull.* 30(1) 242-248). DHEA-S is most stable and crystalline as the dihydrate form. The DHEA-S anhydrous form has low crystallinity and is very hygroscopic. The DHEA-S anhydrous form is stable as long as it picks up no water on storage. Keeping a partially crystalline material free of moisture requires specialized manufacturing and packing technology. For a robust product, minimizing sensitivity to moisture is essential during the development process.

(1) Micronization of DHEA-S

Anhydrous DHEA-S was micronized using a jet milling (Jet-O-Mizer Series #00, 100-120 PSI nitrogen). Approximately 1 g sample was passed through the jet mill, once, and approximately 2 g sample were passed through the jet mill twice. The particles from each milling

Samsung Bioepis Exhibit No. 1002
Page 8346

run were suspended in hexane, in which DHEA-S was insoluble and Spa85 surfactant added to prevent agglomeration. The resulting solution was sonicated for 3 minutes and appeared fully dispersed. The dispersed solutions were tested on a Malvern Mastersizer X with a small volume sampler (SVS) attachment. One sample of dispersed material was tested 5 times. The median particle size or D (v, 0.5) of unmilled material was 52.56 μm and the %RSD (relative standard deviation) was 7.61 for the 5 values. The D (v, 0.5) for a single pass through the jet mill was 3.90 μm and the %RSD was 1.27, and the D (v, 0.5) from a double pass through the jet mill 3.25 μm and the %RSD was 3.10. This demonstrates that DHEA-S can be jet milled to particles of size suitable for inhalation.

(2) HPLC Analysis

-Two vials (A; single-pass; 150 mg) and (B double-pass; 600 mg) of the micronized drug were available for determining drug degradation during jet milling micronization. Weighed aliquots of DHEA-S from vials A and B were compared to a standard solution of unmilled DHEA-S (10 mg/ml) in an acetonitrile-water solution (1:1). The chromatographic peak area for the HPLC assay of the unmilled drug standard solution (10 mg/ml) gave a value of 23,427. Weighed aliquots of micronized DHEA-S form vials A and B, (5 mg/ml) was prepared in an acetonitrile-water solution (1:1). The chromatographic peak areas for vials A and B were 11,979 and 11,677, respectively. Clearly, there was no detectable degradation of the drug during the jet milling micronization process.

(3) Emitted Dose Studies

DHEA-S powder was collected in Nephele tubes and assayed by HPLC. Triplicate experiments were performed at each airflow rate for each of the three dry powder inhalers tested (Rotahaler, Diskhaler and IDL's DPI devices). A Nephele tube was fitted at one end with a glass filter (Gelman Sciences, Type A/E, 25 μm), which in turn was connected to the airflow line to collect the emitted dose of the drug from the respective dry powder inhaler being tested. A silicone adapter, with an opening to receive the mouthpiece of the respective dry powder inhaler being tested at the other end of the Nephele tube was secured. A desired airflow, of 30, 60, or 90 L/min, was achieved through the Nephele tube. Each dry powder inhaler's mouthpiece was inserted then into the silicone rubber adapter, and the airflow was continued for about four s after which the tube was removed and an end-cap screwed onto the end of each tube. The end-cap of the tube not containing the filter was removed and 10 ml of an HPLC grade water- acetonitrile solution (1:1) added to the tube, the end-cap reattached, and the tube shaken for 1-2 minutes. The end-cap then was removed from the tube and the solution was transferred to a 10 ml plastic

Samsung Bioepis Exhibit No. 1002
Page 8347

syringe fitted with a filter (Cameo 13N Syringe Filter, Nylon, 0.22μm). An aliquot of the solution was directly filtered into an HPLC vial for later drug assay via HPLC. The emitted dose experiments were performed with micronized DHEA-S (about 12.5 or 25 mg) being placed in either a gelatin capsule (Rotahaler) or a Ventodisk blister (Diskhaler and single-dose DPI (IDL)). When the micronized DHEA-S (only vial B used), was weighed for placement into the gelatin capsule or blister, there appeared to be a few aggregates of the micronized powder. The results of the emitted dose tests conducted at an airflow rate of 30, 60 and 87.8 L/min are displayed in Tables 2. Table 2 summarizes the results for the Rotahaler experiments at 3 different flow rates, for the Diskhaler experiments at 3 different flow rates, and of the multi-dose experiments at 3 different flow rates.

Samsung Bioepis Exhibit No. 1002
Page 8348

Table 2. Emitted Dose Comparison of Three Different Dry Powder Inhaler Devices

Inhaler Device	Airflow Rate (L/min)	Emitted Dose (%)
Rotahaler	87.8	73.2, 67.1, 68.7
Average		69.7
Rotahaler (2nd study)	87.8	16.0, 24.5, 53.9
Average		31.5
Diskhaler	87.8	65.7, 41.6, 46.5
Average		51.3
Diskhaler (2nd study)	87.8	57.9, 59.9, 59.5
Average		59.1
IDL Multi-Dose	87.8	71.3, 79.0, 67.4
Average		72.6
IDL Multi-Dose (2nd study)	87.8	85.7, 84.6, 84.0
Average		84.8
Rotahaler	60	58.1, 68.2, 45.7
Average		57.3
Diskhaler	60	63.4, 38.9, 58.0
Average		68.2
IDL Multi-Dose	60	78.8, 83.7, 89.6
Average		84.0
Rotahaler	30	34.5, 21.2, 48.5
Average		34.7
Diskhaler	30	53.8, 53.4, 68.7
		58.6
IDL Multi-Dose	30	78.9, 88.2, 89.2
Average		85.4

(4) Respirable Dose Studies

The respirable dose (respirable fraction) studies were performed using a standard sampler cascade impactor (Andersen), consisting of an inlet cone (an impactor pre-separator was substituted here), 9 stages, 8 collection plates, and a backup filter within 8 aluminum stages held together by 3 spring clamps and gasket O-ring seals, where each impactor stage contains multiple precision drilled orifices. When air is drawn through the sampler, multiple jets of air in each stage direct any airborne particles toward the surface of the collection plate for that stage. The size of the jets is constant for each stage, but is smaller in each succeeding stage. Whether a particle is impacted on any given stage depends upon its aerodynamic diameter. The range of particle sizes collected on each stage depends upon on the jet velocity of the stage, and the cut-off point of the

Samsung Bioepis Exhibit No. 1002
Page 8349

previous stage. Any particle not collected on the first stage follows the air stream around the edge of the plate to the next stage, where it is either impacted or passed on to the succeeding stage, and so on, until the velocity of the jet is sufficient for impaction. To prevent particle bounce during the cascade impactor test, the individual impactor plates were coated with a hexane-grease (high vacuum) solution (100:1 ratio). As noted above, the particle size cut-off points on the impactor plates changed at different airflow rates. For example, Stage 2 corresponds to a cut-off value greater than 6.2 μm particles at 60 L/min, and greater than 5.8 μm particles at 30 L/min, and stage 3 had a particle size cut-off value at 90 L/min greater than 5.6 μm. Thus, similar cut-off particle values are preferentially employed at comparable airflow rates, i.e. ranging from 5.6 to 6.2 μm. The set-up recommended by the United States Phamacopeia for testing-dry powder inhalers consists of a mouthpiece adapter (silicone in this case) attached to a glass throat (modified 50 ml round-bottom flask) and a glass distal pharynx (induction port) leading top the pre-separator and Andersen sampler. The pre-separator sample includes washings from the mouthpiece adaptor, glass throat, distal glass pharynx and pre-separator. 5 ml acetonitrile:water (1:1 ratio) solvent was placed in the pre-separator before performing the cascade impactor experiment, that were performed in duplicate with 3 different dry powder inhaler devices and at 3 airflow rates, 30, 60 and 90 L/min. The drug collected on the cascade impactor plates were assayed by the HPLC, and a drug mass balance was performed for each Diskhaler and multi-dose cascade impactor experiment consisting of determining the amount of drug left in the blister, the amount of drug remaining in the device (Diskhaler only), the non-respirable amount of the dose retained on the silicone rubber mouth piece adaptor, glass throat, glass distal pharynx and pre-separator, all combined into one sample, and the respirable dose, i.e. Stage 2 through filter impactor plates for airflow rates of 30 and 60 L/min and Stages 1 through filter impactor plates for 90 L/min experiments.

Samsung Bioepis Exhibit No. 1002
Page 8350

Table 3. Cascade Impactor Experiments (90L/min)

Inhaler Device	Preseparator (%)	Blister (%)	Respirable Dose (%)	Device (%)	Mass Balance (%)
Diskhaler	72.7	6.6	2.9	22.1	104.3
Diskhaler	60.2	10.1	2.4	13.3	86.0
Multi-dose	65.8	3.9	3.8	26.5 *a	100.0
Multi-dose	73.3	3.8	3.6	19.3 *a	100.0
Multi-dose *b	78.7	2.8	4.6	13.9 *a	100.0
Multi-dose *c	55.9	5.0	1.2	37.9 *a	100.0

*a: Multi-dose device was not washed; as solvents would attack SLA components. Multi-dose device retention percentage is obtained by difference.

*b: oven dried drug for 80 minutes

*c: oven dried drug for 20 hours

Based on the results of the emitted dose and cascade impactor experiments, the low respirable dose values achieved in the cascade impactor experiments were due to agglomerated drug particles, which could not be separated, even at the highest airflow rate tested. Agglomeration of the drug particles is a consequence of static charge build up during the mechanical milling process used for particles size reduction and that this situation is further compounded by subsequent moisture absorption of the particles. A micronization method that produces less static charge or a less hygroscopic, fully hydrated crystalline form of DHEA-S (i.e. dihydrate form) should provide a freer flowing powder with diminished potential for agglomeration.

Example 4: Spray Drying of Anhydrous DHEA-S and Determination of Respirable Dose

(1) Micronization of the Drug

1.5 g of anhydrous DHEA-S were dissolved to 100 ml of 50% ethanol:water to produce a 1.5 % solution. The solution was spray-dried with a B-191 Mini Spray-Drier (Buchi, Flawil, Switzerland) with an inlet temperature of 55°C, outlet temperature of 40°C, at 100% aspirator, at 10% pump, nitrogen flow at 40 mbar and spray flow at 600 units. The spray-dried product was suspended in hexane and Span85 surfactant added to reduce agglomeration. The dispersions were sonicated with cooling for 3-5 minutes for complete dispersion and the dispersed solutions tested on a Malvern Mastersizer X with a Small Volume Sampler (SVS) attachment. The two batches of spray dried material were found to have mean particle sizes of 5.07 ±0.70 μm and 6.66 ±0.91

Samsung Bioepis Exhibit No. 1002
Page 8351

μm. Visual examination by light microscope of the dispersions of each batch confirmed that spray drying produced small respirable size particles. The mean particle size was 2.4 μm and 2.0 μm for each batch, respectively. This demonstrates that DHEA-S can be spray dried to a particle size suitable for inhalation.

(2) Respirable Dose Studies

The cascade impactor experiments were conducted as described in Example 3. Four cascade impactor experiments were done, three with a IDL multi-dose device and one with a Diskhaler, all at 90 L/min. The results of the cascade impactor experiments are presented in Table 4 below. The spray-dried anhydrous material in these experiments produced a two-fold increase in the respirable dose compared to micronized anhydrous DHEA-S. It appears that spray drying obtained higher respirable doses as compared to jet-milling. However, the % respirable dose was still low. This was likely the result of moisture absorption of the anhydrous form.

Table 4: Cascade Impactor Results with Spray-Dried Drug Product

Device	Diskhaler	Multi-dose	Multi-dose	Multi-dose
Number of Blisters	3	3	4	4
Drug per Blister (mg)	38.2	36.7	49.4	50.7
Preseparator (%)	56.8	71.9	78.3	85.8
Device (%)	11.2	7.9	8.9	7.6
Blisters (%)	29.0	6.4	8.2	4.8
Respirable Dose (%)	5.6	7.8	5.3	2.6
Mass Balance Recovery (%)	102.7	94.0	103.3	98.1

Example 5: Air Jet Milling of DHEA-S Dihydrate (DHEA-S $\cdot 2H_2O$) and Determination of Respirable Dose

(1) Recrystallization of DHEA-S dihydrate.

Anhydrous DHEA-S is dissolved in a boiling mixture of 90% ethanol/water. This solution is rapidly chilled in a dry ice/methanol bath to recrystallize the DHEA-S. The crystals are filtered, washed twice with cold ethanol, than dried in a vacuum desiccator at RT for 36 h. During the drying process, the material is periodically mixed with a spatula to break large agglomerates. After drying, the material is passed through a 500 μm sieve.

(2) Micronization and physiochecmical testing.

Samsung Bioepis Exhibit No. 1002
Page 8352

DHEA-S dihydrate is micronized with nitrogen gas in a jet mill at a venturi pressure of 40 PSI, a mill pressure of 80 PSI, feed setting of 25 and a product feed rate of about 120 to 175 g/hour. Surface area is determined using five point BET analyses are performed with nitrogen as the adsorbing gas ($P/P_o = 0.05$ to 0.30) using a Micromeritics TriStar surface area analyzer. Particle size distributions are measured by laser diffraction using a Micromeritics Saturn Digisizer where the particles are suspended in mineral oil with sodium dioctyl sodium sulfosuccinate as a dispersing agent. Drug substance water content is measured by Karl Fischer titration (Schott Titroline KF). Pure water is used as the standard and all relative standard deviations for triplicates are less than 1%. Powder is added directly to the titration media. The physicochemical properties of DHEA-S·dihydrate before and after micronization are summarized in Table 5.

Table 5. Physicochemical properties of DHEA-S·dihydrate before and after micronization.

Property	Bulk	Micronized
Particle size ($D_{50\%}$)	31 microns	3.7 microns
Surface area (m^2/g)	Not measured	4.9
Water (% w/w)	8.5	8.4
Impurities	No significant peaks	No significant peaks

The only significant change measured is in the particle size. There is no significant loss of water or increase in impurities. The surface area of the micronized material is in agreement with an irregularly shaped particle having a median size of 3 to 4 microns. The micronization successfully reduces the particle size to a range suitable for inhalation with no measured changes in the solid-state chemistry.

(3) Aerosolization of DHEA-S·dihydrate.

The single-dose Acu-Breathe device is used for evaluating DHEA-S·dihydrate. Approximately 10 mg of neat DHEA-S·dihydrate powder is filled and sealed into foil blisters. These blisters are actuated into the Andersen 8-stage cascade impactor at flow rates ranging from 30 to 75 L/min with a glass twin-impinger throat. Stages 1-5 of the Andersen impactor are rinsed together to obtain an estimate of the fine particle fraction. Pooling the drug collected from multiple stages into one assay make the method much more sensitive. The results for this series of experiments is shown in Figure 1. At all flow rates, the dihydrate yields a higher fine particle fraction than the virtually anhydrous material. Since the dihydrate powder is aerosolized using the single-dose inhaler, it is very reasonable to conclude that its aerosol properties are

Samsung Bioepis Exhibit No. 1002
Page 8353

significantly better than the virtually anhydrous material. Higher crystallinity and stable moisture content are the most likely factors contributing the dihydrate's superior aerosol properties. This unique feature of DHEA-S·dihydrate has not been reported in any previous literature. While the improvement in DHEA-S's aerosol performance with the dihydrate form is significant, neat drug substance may not be the optimal formulation. Using a carrier with a larger particle size typically improves the aerosol properties of micronized drug substances.

Example 6: Anhydrous DHEA-S and DHEA-S Dihydrate Stability with and without Lactose

The initial purity (Time=0) was determined for anhydrous DHEA and for DHEA-S dihydrate by high pressure liquid chromatography (HPLC). Both forms of DHEA-S were then either blended with lactose at a ratio of 50:50, or used as a neat powder and placed in open glass vials, and held at 50°C for up to 4 weeks. These conditions were used to stress the formulation in order to predict its long-term stability results. Control vials containing only DHEA-S (anhydrous or dihydrate) were sealed and held 25°C for up to 4 weeks. Samples were taken and analyzed by HPLC also at 0, 1, 2, and 4 weeks to determine the amount of degradation, as determined by formation of DHEA. After one week, virtually anhydrous DHEA-S blended with lactose (50% w/w, nominally) stored at 50°C in sealed glass vials acquires a brown tinge that is darker for the lactose blend. This color change is accompanied by a significant change in the chromatogram as shown in Figure 1. The primary degradant is DHEA. Qualitatively from Figure 2, the amount of DHEA in the blend is higher than the other two samples. To quantitatively estimate the % DHEA in the samples, the area for the DHEA peak is divided by the total area for the DHEA-S and DHEA peaks (see Table 6). The higher rate of decomposition for the blend indicates a specific interaction between lactose and the virtually anhydrous DHEA-S. In parallel with the increase in DHEA, the brown color of the powders on accelerated storage increased over time. The materials on accelerated storage become more cohesive with time as evidenced by clumping during sample weighing for chemical analysis. Based on these results, it is not possible to formulate virtually anhydrous DHEA-S with lactose. This is a considerable disadvantage since lactose is the most commonly used inhalation excipient for dry powder formulations. Continuing with the virtually anhydrous form would mean limiting formulations to neat powder or undertaking more comprehensive safety studies to use a novel excipient.

Table 6: DHEA % formed from Anhydrous DHEA-S at 50°C

Samsung Bioepis Exhibit No. 1002
Page 8354

Formulation	Time (Weeks)	1	2	4
Control	2.774	2.694	2.370	2.666
DHEA-S. Alone		9.817	14.954	20.171
DHEA-S+Lactose (50:50)		24.085	30.026	38.201

In contrast to Figure 2, there is virtually no DHEA generated after storage for 1 week at 50 °C (see Figure 3). Furthermore, the materials show no change in color. The moisture content of DHEA-S·dihydrate remains virtually unchanged after one week at 50 °C. The water content after accelerated storage is 8.66% versus a starting value of 8.8%. The %DHEA measured during the course of this stability program is shown in Table 7.

Table 7: Percent DHEA formed from DHEA-S Dihydrate at 50°C

Formulation	Time (Weeks)	1	3	4
Control	0.213			0.218
DHEA-S alone		0.216	0.317	0.374
DHEA-S:Lactose (50:50)		0.191	0.222	0.323

By comparing Figures 1 and 2 and Tables 6 and 7, one can see that the dihydrate form of DHEA-S is the more stable form for progression into further studies. The superior compatibility of DHEA-S·dihydrate with lactose over that of the virtually anhydrous material has not been reported in the patent or research literature. The solubility of this substance is reported in the next section as a portion of the development work for a nebulizer solution.

Example 7: DHEA-S Dihydrate/Lacotse blends, Determination of Respirable Dose and Stability

(1) DHEA-S dihydrate/Lactose blend.

Equal weights of DHEA-S and inhalation grade lactose (Foremost Aero Flo 95) are mixed by hand then passed through a 500 μm screen to prepare a pre-blend. The pre-blend is then placed in a BelArt Micro-Mill with the remaining lactose to yield a 10% w/w blend of

Samsung Bioepis Exhibit No. 1002
Page 8355

DHEA-S. The blender is wired to a variable voltage source to regulate the impeller speed. The blender voltage is cycled through 30%, 40%, 45% and 30% of full voltage for 1, 3, 1.5, and 1.5 minutes, respectively. The content uniformity of the blend was determined by HPLC analysis. Table 8 shows the result of content uniformity samples for this blend. The target value is 10% w/w DHEA-S. The blend content is satisfactory for proximity to the target value and content uniformity.

Table 8: Content uniformity for a blend of DHEA-S·dihydrate with lactose.

Sample	% DHEA-S, w/w
1	10.2
2	9.7
3	9.9
4	9.3
5	9.4
Mean	9.7
RSD	3.6%

(2) Aerosolization of DHEA-S·dihydrate/Lactose blend.

Approximately 25 mg of this powder is filled and sealed in foil blisters and aerosolized using the single-dose device at 60 L/min. Two blisters are used for each test and the results for fine particle fraction (material on stages 1-5) are shown in Table 9. The aerosol results for this preliminary powder blend are satisfactory for a respiratory drug delivery system. Higher fine particle fractions are possible with optimization of the powder blend and blister/device configuration. The entire particle size distribution of Test 2 is shown in Table 10. This median diameter for DHEA-S for this aerosol is ~2.5 μm. This diameter is smaller than the median diameter measured for micronized DHEA-S·dihydrate by laser diffraction. Irregularly shaped particles can behave aerodynamically as smaller particles since their longest dimension tends to align with the air flow field. Therefore, it is common to see a difference between the two methods. Diffraction measurements are a quality control test for the input material while cascade impaction is a quality control test for the finished product.

Table 9: Fine particle fraction for lactose blend in two different experiments

Samsung Bioepis Exhibit No. 1002
Page 8356

Test	Total powder weight in two blisters (mg)	DHEA-S collected Stages 1-5 (mg)	Fine particle fraction, %
1	52.78	1.60	31
2	57.09	1.62	29

Table 10: Particle size distribution of aerosolized DHEA-S dihydrate/Lactose Blend

Size (μm)	6.18	9.98	3.23	2.27	1.44	0.76	0.48	0.27
% Particles Under	100	87.55	67.79	29.87	10.70	2.57	1.82	0.90

(3) Stability of DHEA-S Dihydrate/Lactose Blend.

This lactose formulation is also placed on an accelerated stability program at 50°C. The results for DHEA-S content are in Table 11. The control is the blend stored at RT. There is no trend in the DHEA-S content over time for either condition and all the results are within the range of samples collected for content uniformity testing (see Table 11). Furthermore, there are no color changes or irregularities observed in the chromatograms. The blend appears to be chemically stable.

Table 11: Stressed stability data on DHEA-S·dihydrate/lactose blend at 50°C.

Time (weeks)	% DHEA-S w/w for control condition	% DHEA-S w/w for stressed condition
0	9.7	9.7
1	9.6	9.6
1.86	9.5	9.7
3	10	9.9

Example 8: Nebulizer Formulation of DHEA-S

Solubility of DHEA-S.

An excess of DHEA-S dihydrate, prepared according to "Recrystallization of DHEA-S·Dihydrate (Example 5)", is added to the solvent medium and allowed to equilibrate for at least 14 hours with some periodic shaking. The suspensions are then filtered through a 0.2 micron syringe filter and immediately diluted for HPLC analysis. To prepare refrigerated samples, the syringes and filters are stored in the refrigerator for at least one hour before use. Inhalation of pure water can produce a cough stimulus. Therefore, it is important to add halide ions to a

Samsung Bioepis Exhibit No. 1002
Page 8357

nebulizer formulation with NaCl being the most commonly used salt. Since DHEA-S is a sodium salt, NaCl could decrease solubility due to the common ion effect. The solubility of DHEA-S at RT (24-26 °C) and refrigerated (7-8 °C) as a function of NaCl concentration is shown in Figure 4. DHEA-S's solubility decrease with NaCl concentration. Lowering the storage temperature decrease the solubility at all NaCl concentrations. The temperature effect is weaker at high NaCl concentrations. For triplicates, the solubility at ~25 °C and 0% NaCl range from 16.5-17.4 mg/mL with a relative standard deviation of 2.7%. At 0.9% NaCl refrigerated, the range for triplicates is 1.1-1.3 mg/mL with a relative standard deviation of 8.3%.

The equilibrium between DHEA-S in the solid and solution states is:

$$NaDHEA\text{-}S_{solid} \leftrightarrow DHEA\text{-}S^- + Na^+$$

$$K = [DHEA\text{-}S^-][Na^+]/[NaDHEA\text{-}S]_{solid}$$

Since the concentration of DHEA-S in the solid is constant (i.e., physically stable dihydrate), the equilibrium expression is simplified:

$$Ksp = [DHEA\text{-}S^-][Na^+]$$

Based on this presumption, a plot of DHEA-S solubility versus the reciprocal of the total sodium cation concentration is linear with a slope equal to Ksp. This is shown in Figures 5 and 6 for equilibrium at RT and refrigerated, respectively. Based on the correlation coefficients, the model is a reasonable fit to the data at both room and refrigerated temperatures where the equilibrium constants were 2236 and 665 mM^2, respectively. To maximize solubility, the NaCl level needs to be as low as possible. The minimum halide ion content for a nebulizer solution should be 20 mM or 0.12% NaCl.

To estimate a DHEA-S concentration for the solution, a 10 °C temperature drop in the nebulizer during use is assumed (i.e., 15 °C). Interpolating between the equilibrium constants versus the reciprocal of absolute temperature, the Ksp at 15 °C would be ~ 1316 mM^2. Each mole of DHEA-S contributes a mole of sodium cation to the solution, therefore:

$$Ksp = [DHEA\text{-}S^-][Na^+]=[DHEA\text{-}S^-][Na^+ + DHEA\text{-}S^-]$$

$$= [DHEA\text{-}S^-]^2 + [Na^+][DHEA\text{-}S^-]$$

which is solve for $[DHEA\text{-}S^-]$ using the quadratic formula. The solution for 20 mM Na^+ with a Ksp of 1316 mM^2 is 27.5 mM $DHEA\text{-}S^-$ or 10.7 mg/mL. Therefore a 10 mg/mL DHEA-S solution in 0.12% NaCl is selected as a good candidate formulation to progress into additional testing. The estimate for this formula does not account for any concentration effects due to water evaporation from the nebulizer. The pH of a 10 mg/mL DHEA-S solution with 0.12% NaCl range from 4.7 to 5.6. While this would be an acceptable pH level for an inhalation

Samsung Bioepis Exhibit No. 1002
Page 8358

formulation, the effect of using a 20 mM phosphate buffer is evaluated. The solubility results at RT for buffered and unbuffered solutions are shown in Figure 7. The presence of buffer in the formulation suppress the solubility, especially at low NaCl levels. As shown in Figure 8, the solublity data for the buffered solution falls on the same equilibrium line as for the unbuffered solution. The decrease in solubility with the buffer is due to the additional sodium cation content. Maximizing solubility is an important goal and buffering the formulation reduces solubility. Furthermore, Ishihora and Sugimoto ((1979) *Drug Dev. Indust. Pharm.* 5(3) 263-275) did not show a significant improvement in NaDHEA-S stability at neutral pH.

Stability Studies.

A 10 mg/mL DHEA-S formulation is prepared in 0.12% NaCl for a short-term solution stability program. Aliquots of this solution are filled into clear glass vials and stored at RT (24-26 °C) and at 40 °C. The samples are checked daily for DHEA-S content, DHEA content, and appearance. For each time point, duplicate samples are withdrawn and diluted from each vial. The DHEA-S content over the length of this study is shown in Figures 9 and 10. At the accelerated condition, the solution show a faster decomposition rate and became cloudy after two days of storage. The solution stored at RT is more stable and a slight precipitate is observed on the third day. The study is stopped on day three. DHEA-S decomposition is accompanied by an increase in DHEA content as shown in Figure 10. Since DHEA is insoluble in water, it only takes a small quantity in the formulation to create a cloudy solution (accelerated storage) or a crystalline precipitate (room storage). This explains why earlier visual evaluations of DHEA-S solubility severely underestimate the compound's solubility: small quantities of DHEA would lead the experimenter to conclude the solubility limit of DHEA-S had been exceeded. The solution should easily be stable for the day of reconstitution in a clinical trial. The following section describes the aerosol properties of this formulation.

Nebulizer Studies.

DHEA-S solutions are nebulized using a Pari ProNeb Ultra compressor and LC Plus nebulizer. The schematic for the experiment set-up is shown in Figure 11. The nebulizer is filled with 5 mL of solution and nebulization is continued until the output became visually insignificant (4½ to 5 min.). Nebulizer solutions are tested using a California Instruments AS-6 6-stage impactor with a USP throat. The impactor is run at 30 L/min for 8 s to collect a sample following one minute of nebulization time. At all other times during the experiment, the aerosol is drawn through the by-pass collector at approximately 33 L/min. The collection apparatus, nebulizer, and impactor are rinsed with mobile phase and assayed by HPLC. 5 mL of DHEA-S

Samsung Bioepis Exhibit No. 1002
Page 8359

in 0.12% NaCl is used in the nebulizer. This volume is selected as the practical upper limit for use in a clinical study. The results for the first 5 nebulization experiments are shown below:

Table 12. Results for nebulization studies with DHEA-S

Solution-Nebulizer #	Left in Nebulizer, mg	Deposited in Collector, mg	Deposited in Impactor, mg	Total, mg
10 mg/mL-1	17.9*	16.3	0.38	34.6
10 mg/mL-2	31.2	17.2	0.48	49.0
7.5 mg/mL-1	19.3	16.3	0.35	36.0
7.5 mg/mL-1	21.7	15.4	0.30	37.4
5.0 mg/mL-1	14.4	10.6	0.21	25.2

* Only assayed liquid poured from nebulizer; did not weigh before and after aerosolization or rinse entire unit

Nebulizer #1 runs to dryness in about 5 minutes while Nebulizer #2 takes slightly less than 4.5 minutes. In each case, the liquid volume remaining in the nebulizer is approximately 2 mL. This liquid is cloudy initially after removal from the nebulizer then clears within 3-5 minutes. Even after this time, the 10 mg/mL solutions appear to have a small amount of coarse precipitate in them. Fine air bubbles in the liquid appear to cause the initial cloudiness. DHEA-S appears to be surface active (i.e., promoting foam) and this stabilizes air bubbles within the liquid. The precipitate in 10 mg/mL solutions indicates that the drug substance's solubility is exceeded in the nebulizer environment. Therefore, the additional nebulization experiments in Table 13 are run at lower concentrations. able 13 presents additional data of "dose" linearity versus solution concentration.

Table 13. Results from additional nebulizer experiments with DHEA-S.

Solution-Nebulizer #	Left in Nebulizer, mg	Deposited in Collector, mg	Deposited in Impactor, mg	Total, mg
6.25 mg/mL-2	17.8	12.1	0.24	30.1
7.5 mg/mL-3	21.2	13.8	0.33	35.3

Nebulizer #3 takes slightly less than 4.5 minutes to reach dryness. The mass in the by-pass collector is plotted versus the initial solution concentration in Figure 12. There is good linearity from 0 to 7.5 mg/mL then the amount collected appears to start leveling-off. While the

Samsung Bioepis Exhibit No. 1002
Page 8360

solubility reduction by cooling is included in the calculation of the 10 mg/mL solution, any concentration effects on drug and NaCl content were neglected. Therefore, it is possible for a precipitate to form via supersaturation of the nebulizer liquid. The data in Figure 12 and the observation of some particulates in the 10 mg/mL solution following nebulization indicate that the highest solution concentration for a proof of concept clinical trial formulation is approximately 7.5 mg/mL. An aerosol sample is drawn into a cascade impactor for particle size analysis. There is no detectable trend in particle size distribution with solution concentration or nebulizer number. The average particle size distribution for all nebulization experiments is shown in Figure 13. The aerosol particle size measurements are in agreement with published/advertised results for this nebulizer (i.e., median diameter ~2 μm). While the *in vitro* experiments demonstrate that a nebulizer formulation can deliver respirable DHEA-S aerosols, the formulation is unstable and takes 4-5 minutes of continuous nebulization. Therefore, a stable DPI formulation has significant advantages. DHEA-S·dihydrate is identified as the most stable solid state for a DPI formulation. An optimal nebulizer formulation is 7.5 mg/mL of DHEA-S in 0.12% NaCl for clinical trials for DHEA-S. The pH of the formulation is acceptable without a buffer system. The aqueous solubility of DHEA-S is maximized by minimizing the sodium cation concentration. Minimal sodium chloride levels without buffer achieve this goal. This is the highest drug concentration with 20 mM of Cl^- that will not precipitate during nebulization. This formulation is stable for at least one day at RT.

Example 9: Preparation of the Experimental Model

Cell cultures, HT-29 SF cells, which represent a subline of HY-29 cells (ATCC, Rockville, Md.) and are adapted for growth in completely defined serum-free PC-1 medium (Ventrex, Portland, ME), were obtained. Stock cultures were maintained in this medium at 37°C (in a humidified atmosphere containing 5% CO_2). At confluence cultures were replated after dissociation using trypsin/EDTA (Gibco, Grand Island, NY) and re-fed every 24 hours. Under these conditions, the doubling time for HT-29 SF cells during logarithmic growth was 24 hours.

Flow Cytometry

Cells were plated at 10^5/60-mm dish in duplicate. For analysis of cell cycle distribution, cultures were exposed to 0, 25, 50, or 200 μM DHEA. For analysis of reversal of cell cycle effects of DHEA, cultures were exposed to either 0 or 25 μM DHEA, and the media were supplemented with MVA, CH, RN, MVA plus CH, or MVA plus CH plus RN or were not supplemented. Cultures were trypsinized following 0, 24, 48, or 74 hours and fixed and stained

Samsung Bioepis Exhibit No. 1002
Page 8361

using a modification of a procedure of Bauer et al., *Cancer Res.* 46, 3173-3178 (1986). Briefly, cells were collected by centrifugation and resuspended in cold phosphate-buffered saline. Cells were fixed in 70% ethanol, washed, and resuspended in phosphate-buffered saline. One ml hypotonic stain solution (50 μg/ml propidium iodide (Sigma Chemical Co.), 20 μg/ml Rnase A (Boehringer Mannheim, Indianapolis, IN), 30 mg/ml polyethylene glycol, 0.1% Triton X-100 in 5 mM citrate buffer) was then added, and after 10 min at room temperature, 1 ml of isotonic stain solution (propidium iodide, polyethylene glycol, Triton X-100 in 0.4M NaCl) was added and the cells were analyzed using a flow cytometer, equipped with pulse width/pulse area doublet discrimination (Becton Dickinson Immunocytometry Systems, San Jose, CA) After calibration with fluorescent beads, a minimum of $2x10^4$ cells/sample were analyzed, data were displayed s total number of cells in each of 1024 channels of increasing fluorescence intensity, and the resulting histogram was analyzed using the Cellfit analysis program (Becton Dickinson).

DHEA Effect on Cell Growth

Cells were plated 25,000 cells/30 mm dish in quadruplicate, and after 2 days received 0, 12.5, 25, 50, or 200 μM DHEA. Cell number was determined 0, 24, 48, and 72 hours later using a Coulter counter (model Z; Coulter Electronics, Inc. Hialeah, FL). DHEA (AKZO, Basel, Switzerland) was dissolved in dimethyl sulfoxide, filter sterilized, and stored at -20°C until use.

Figure 14 illustrates the inhibition of growth for HT-29 cells by DHEA. Points refer to numbers of cells, and bars refer to SEM. Each data point was performed in quadruplicate, and the experiment was repeated three times. Where SEM bars are not apparent, SEM was smaller than symbol. Exposure to DHEA resulted in a reduced cell number compared to controls after 72 hours in 12.5 μM, 48 hours in 25 or 50 μM, and 24 hours in 200 μM DHEA, indicating that DHEA produced a time- and dose-dependent inhibition of growth.

DHEA Effect on Cell Cycle

To examine the effects of DHEA on cell cycle distribution, HT-29 SF cells were plated (10^5 cells/60 mm dish), and 48 hours later treated with 0,25, 50, or 200 μM DHEA. Fig. 15 illustrates the effects of DHEA on cell cycle distribution in HT-29 SF cells. After 24, 48, and 72 hours, cells were harvested, fixed in ethanol, and stained with propidium iodide, and the DNA content/cell was determined by flow cytometric analysis. The percentage of cells in G_1, S, and G_2M phases was calculated using the Cellfit cell cycle analysis program. S phase is marked by a quadrangle for clarity. Representative histograms from duplicate determinations are shown. The experiment was repeated three times.

Samsung Bioepis Exhibit No. 1002
Page 8362

The cell cycle distribution in cultures treated with 25 or 50 μM DHEA was unchanged after the initial 24 hours. However, as the time of exposure to DHEA increased, the proportion of cells in S phase progressively decreased, and the percentage of cells in G_1, S and G_2M phases was calculated using the Cellfit cell cycle analysis program. S phase is marked by a quadrangle for clarity. Representative histograms from duplicate determinations are shown. The experiment was repeated three times.

The cell cycle distribution in cultures treated with 25 or 50 μM DHEA was unchanged after the initial 24 hours. However, as the time of exposure to DHEA increased, the proportion of cells in S phase progressively decreased and the percentage of cells in G_1 phase was increased after 72 hours. A transient increase in G_2M phase cells was apparent after 48 hours. Exposure to 200μM DHEA produced a similar but more rapid increase in the percentage of cells in G_1 and a decreased proportion of cells in S phase after 24 hours, which continued through the treatment. This indicates that DHEA produced a G_1 block in HT-29 SF cells in a time-and dose-dependent manner.

Example 10: Reversal of DHEA-mediated Effect on Growth and Cell Cycle

Reversal of DHEA-mediated Growth Inhibition.

Cells were plated as above, and after 2 days received either 0 or 25 μM DHEA-containing medium supplemented with mevalonic acid ("MVA"; mM) squalene (SQ; 80 μM), cholesterol (CH; 15 μg/ml), MVA plus CH, ribonucleosides (RN; uridine, cytidine, adenosine, and guanosine at final concentrations of 30 μM each), deoxyribonucleosides (DN; thymidine, deoxycytidine, deoxyadenosine and deoxyguanosine at final concentrations of 20 μM each). RN plus DN, or MVA plus CH plus RN, or medium that was not supplemented. All compounds were obtained from Sigma Chemical Co. (St. Louis, MO) Cholesterol was solubilized in ethanol immediately before use. RN and DN were used in maximal concentrations shown to have no effects on growth in the absence of DHEA.

Figure 16 illustrates the reversal of DHEA-induced growth inhibition in HT-29 SF cells. In A, the medium was supplemented with 2 μM MVA, 80 μM SQ, 15 μg/ml CH, or MVA plus CH (MVA+CH) or was not supplemented (CON). In B, the medium was supplemented with a mixture of RN containing uridine, cytidine, adenosine, and guanosine in final concentrations of 30 μM each; a mixture of DN containing thymidine, deoxycytidine, deoxyadenosine and deoxyguanosine in final concentrations of 20 μM each; RN plus DN (RN+DN); or MVA plus CH plus RN (MVA+CH+RN). Cell numbers were assessed before and after 48 hours of treatment, and culture growth was calculated as the increase in cell number during the 48 hour treatment

Samsung Bioepis Exhibit No. 1002
Page 8363

period. Columns represent cell growth percentage of untreated controls; bars represent SEM. Increase in cell number in untreated controls was 173,370"6518. Each data point represents quadruplicate dishes from four independent experiments. Statistical analysis was performed using Student's t test κ $p<0.01$; ψ p<, 0.001; compared to treated controls. Note that supplements had little effect on culture growth in absence of DHEA.

Under these conditions, the DHEA-induced growth inhibition was partially overcome by addition of MVA as well as by addition of MVA plus CH. Addition of SQ or CH alone had no such effect. This suggest that the cytostatic activity of DHEA was in part mediated by depletion of endogenous mevalonate and subsequent inhibition of the biosynthesis of an early intermediate in the cholesterol pathway that is essential for cell growth. Furthermore, partial reconstitution of growth was found after addition of RN as well as after addition of RN plus DN but not after addition of DN, indicating that depletion of both mevalonate and nucleotide pools is involved in the growth-inhibitory action of DHEA. However, none of the reconstitution conditions including the combined addition of MVA, CH, and RN completely overcame the inhibitory action of DHEA, suggesting either cytotoxic effects or possibly that additional biochemical pathways are involved.

Reversal of DHEA Effect on Cell Cycle

HT-29 SF cells were treated with 25 FM DHEA in combination with a number of compounds, including MVA, CH, or RN, to test their ability to prevent the cell cycle-specific effects of DHEA. Cell cycle distribution was determined after 48 and 72 hours using flow cytometry.

Figure 17 illustrates reversal of DHEA-induced arrest in HT-29 SF cells. Cells were plated (10^5 cells/60 mm dish) and 48 hours later treated with either 0 or 25 FM DHEA. The medium was supplemented with 2 FM MVA; 15 Fg/ml CH; a mixture of RN containing uridine, cytidine, adenosine, and guanosine in final concentrations of 30 FM; MVA plus CH (MVA+CH); or MVA plus CH plus RN (MVA+CH+RN) or was not supplemented. Cells were harvested after 48 or 72 hours, fixed in ethanol, and stained with propidium iodine, and the DNA content per cell was determined by flow cytometric analysis. The percentage of cells in G_1, S, and G_2M phases were calculated using the Cellfit cell cycle profile analysis program. S phase is marked by a quadrangle for clarity. Representative histograms from duplicative determinations are shown. The experiment was repeated two times. Note that supplements had little effect on cell cycle progression in the absence of DHEA.

With increasing exposure time, DHEA progressively reduced the proportion of cells in S

Samsung Bioepis Exhibit No. 1002
Page 8364

phase. While inclusion of MVA partially prevented this effect in the initial 48 hours but not after 72 hours, the addition of MVA plus CH was also able to partially prevent S phase depletion at 72 hours, suggesting a requirement of both MVA and CH for cell progression during prolonged exposure. The addition of MVA, CH, and RN was apparently most effective at reconstitution but still did not restore the percentage of S phase cells to the value seen in untreated control cultures. CH or RN alone had very little effect at 48 hours and no effect at 72 hours. Morphologically, cells responded to DHEA by acquiring a rounded shape, which was prevented only by the addition of MVA to the culture medium. Some of the DNA histograms after 72 hours DHEA exposure in FIG.4 also show the presence of a subpopulation of cells possessing apparently reduced DNA content. Since the HT-29 cell line is known to carry populations of cells containing varying numbers of chromosomes (68-72; ATCC), this may represent a subset of cells that have segregated carrying fewer chromosomes.

Conclusions

The Examples 9-10 above provide evidence that in vitro exposure of HT-29 SF human colonic adenocarcinoma cells to concentrations of DHEA known to deplete endogenous mevalonate results in growth inhibition and G_1 arrest and that addition of MVA to the culture medium in part prevents these effects. DHEA produced effects upon protein isoprenylation which were in many respects similar to those observed for specific 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors such as lovastatin and compactin. Unlike direct inhibitors of mevalonate biosynthesis, however, DHEA mediates its effects upon cell cycle progression and cell growth in a pleiotropic manner involving ribo-and deoxyribonucleotide biosynthesis and possibly other factors as well.

Example 11: Metered Dose Inhaler

Active Ingredient	Target per Actuation
Omalizumab	10.0 mg
DHEA	400 mg
Stabilizer	5.0 µg
Trichlorofluoromethane	23.70 mg
Dichlorodifluoromethane	61.25 mg

Example 12: Metered Dose Inhaler

Active Ingredient	Target per Actuation

Samsung Bioepis Exhibit No. 1002
Page 8365

Omalizumab	10.0 mg
DHEA-S	400 mg
Stabilizer	7.5 µg
Trichlorofluoromethane	23.67 mg
Dichlorodifluoromethane	61.25 mg

In the following Examples 13 and 14, the first and second active agents are micronized and bulk blended with lactose in the proportions given above. The blend is filled into hard gelatin capsules or cartridges or into specifically constructed double foil blister packs (Rotadisks blister packs, Glaxo®) to be administered by an inhaler such as the Rotahaler inhaler (Glaxo®) or in the case of the blister packs with the Diskhaler inhaler (Glaxo®).

Example 13: Metered Dose Dry Powder Formulation

Active Ingredient	/cartridge or blister
Omalizumab	10.0 mg
DHEA	1.00 mg
Lactose Ph. Eur.	to 12.5 or 25.0 mg

Example 14: Metered Dose Dry Powder Formulation

Active Ingredient	/cartridge or blister
Omalizumab	10.0 mg
DHEA-S	1.00 mg
Lactose Ph. Eur.	to 12.5 or 25.0 mg

Although the invention has been described with reference to the presently preferred embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention.

Samsung Bioepis Exhibit No. 1002
Page 8366

All publications, patents, and patent applications, and web sites are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent, or patent application, was specifically and individually indicated to be incorporated by reference in its entirety.

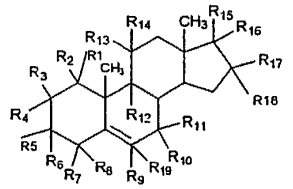
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Samsung Bioepis Exhibit No. 1002
Page 8367

WHAT IS CLAIMED IS:

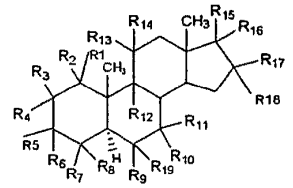
1. A pharmaceutical composition, comprising a pharmaceutically or veterinarily acceptable carrier, a first active agent and a second active agent effective to treat asthma, chronic obstructive pulmonary disease, or a respiratory or lung disease,

(a) the first active agent is at least one of a non-glucocorticoid steroid selected from a non-glucocorticoid steroid having the chemical formula



(III)

and a non-glucocorticoid steroid of the chemical formula



(IV)

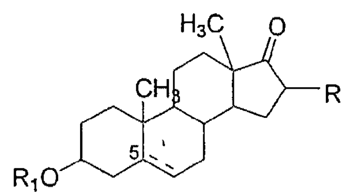
wherein R1, R2, R3, R4. R5, R7, R8, R9, R10, R12, R13, R14 and R19 are independently H, OR, halogen, (C1-C10) alkyl or (C1-C10) alkoxy, R5 and R11 are independently OH, SH, H, halogen, pharmaceutically acceptable ester, pharmaceutically acceptable thioester, pharmaceutically acceptable ether, pharmaceutically acceptable thioether, pharmaceutically acceptable inorganic esters, pharmaceutically acceptable monosaccharide, disaccharide or oligosaccharide, spirooxirane, spirothirane, -OSO2R20, -OPOR20R21 or (C1-C10) alky, R5 and R6 taken together are =O, R10 and R11 taken together are =O; R15 is (1) H, halogen, (C1-C10) alkyl, or (C1-C10) alkoxy when R16 is -C(O)OR22, (2) H, halogen, OH or (C1-C10) alkyl when R16 is halogen, OH or (C1-C10) alkyl, (3) H, halogen, (C1-C10) alkyl, (C1-C10) alkenyl, (C1-C10) alkynyl, formyl, (C1-C10) alkanoyl or epoxy when R16 is OH, (4) OR, SH, H, halogen, pharmaceutically acceptable ester, pharmaceutically acceptable thioester, pharmaceutically acceptable ether, pharmaceutically acceptable thioether, pharmaceutically acceptable inorganic esters, pharmaceutically acceptable monosaccharide, disaccharide or oligosaccharide, spirooxirane, spirothirane, -OSO2R20 or -OPOR20R21 when R16 is H, or R15 and R16 taken together are =O; R17 and R18 are independently (1) H, -OH, halogen, (C1-C10) alkyl or -(C1-C10) alkoxy when R6 is H OR, halogen. (C1-C10) alkyl or -C(O)OR22, (2) H, (C1-C10 alkyl).amino, ((C1-C10) alkyl)n amino-(C1-C10) alkyl, (C1-C10) alkoxy, hydroxy - (C1-C10)

Samsung Bioepis Exhibit No. 1002
Page 8368

alkyl, (C1-C10) alkoxy - (C1-C10) alkyl, (halogen)m (C1-C10) alkyl, (C1-C10) alkanoyl, formyl, (C1-C10) carbalkoxy or (C1-C10) alkanoyloxy when R15 and R16 taken together are =O, (3) R17 and R18 taken together are =O; (4) R17 or R18 taken together with the carbon to which they are attached form a 3-6 member ring containing 0 or 1 oxygen atom; or (5) R15 and R17 taken together with the carbons to which they are attached form an epoxide ring; R20 and R21 are independently OH, pharmaceutically acceptable ester or pharmaceutically acceptable ether; R22 is H, (halogen)m (C1-C10) alkyl or (C1-C10) alkyl; n is 0, 1 or 2; and m is 1, 2 or 3; or pharmaceutically or veterinarily acceptable salts thereof; and

(b) the second active agent is an anti-IgE antibody.

2. The pharmaceutical composition of claim 1 wherein said first active agent is a non-glucocorticoid steroid having the chemical formula



(I)

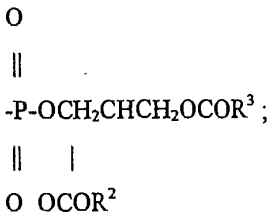
wherein the broken line represents a single or a double bond; R is hydrogen or a halogen; the H at position 5 is present in the alpha or beta configuration or the compound of chemical formula I comprises a racemic mixture of both configurations; and R^1 is hydrogen or a multivalent inorganic or organic dicarboxylic acid covalently bound to the compound.

3. The pharmaceutical composition of claim 1, wherein the first active agent is a non-glucocorticoid steroid having the chemical formula (I), wherein said multivalent organic dicarboxylic acid is SO_2OM, phosphate or carbonate, wherein M comprises a counterion, wherein said counterion is H, sodium, potassium, magnesium, aluminum, zinc, calcium, lithium, ammonium, amine, arginine, lysine, histidine, triethylamine, ethanolamine, choline, triethanoamine, procaine, benzathine, tromethanine, pyrrolidine, piperazine, diethylamine, sulfatide

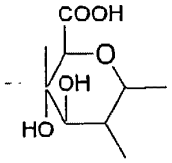
$$-SO_2O\text{-}CH_2CHCH_2OCOR^3;$$
$$|$$
$$OCOR^2$$

Samsung Bioepis Exhibit No. 1002
Page 8369

or phosphatide



wherein R^2 and R^3, which are the same or different, and are straight or branched (C_1-C_{14}) alkyl or glucuronide

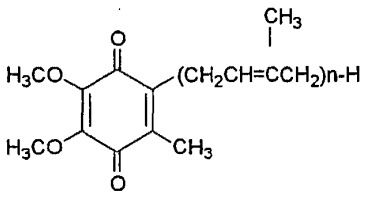


4. The pharmaceutical composition of claim 3, wherein said first active agent is dehydroepiandrosterone.

5. The pharmaceutical composition of claim 3, wherein said first active agent is dehydroepiandrosterone-sulfate.

6. The pharmaceutical composition of claim 1, wherein said anti-IgE antibody is E-25 (omalizumab).

7. The pharmaceutical composition of claim 1, further comprising a ubiquinone or pharmaceutically or veterinarily acceptable salt thereof, wherein the ubiquinone has the chemical formula



(II)

wherein n is 1 to 12.

Samsung Bioepis Exhibit No. 1002
Page 8370

8. The pharmaceutical composition of claim 1, wherein the pharmaceutical composition comprises particles of inhalable or respirable size.

9. The pharmaceutical composition of claim 8, wherein the particles are about 0.01 μm to about 10 μm in size.

10. The pharmaceutical composition of claim 8, wherein the particles are about 10 μm to about 100 μm in size.

11. A kit comprising a delivery device and the pharmaceutical composition of claim 1.

12. The kit of claim 11, wherein the delivery device is an aerosol generator or spray generator.

13. The kit of claim 12, wherein the aerosol generator comprises an inhaler.

14. The kit of claim 13, wherein the inhaler delivers individual pre-metered doses of the formulation

15. The kit of claim 13, wherein the inhaler comprises a nebulizer or insufflator.

16. A method for reducing the probability of or treating asthma in a subject, comprising administering to a subject in need of such treatment a prophylactically or therapeutically effective amount of the pharmaceutical composition of claim 1.

17. A method for reducing the probability of or treating of chronic obstructive pulmonary disease in a subject, comprising administering to a subject in need of such treatment a prophylactically or therapeutically effective amount of the pharmaceutical composition of claim 1.

18. A method for treatment of respiratory, lung or malignant disorder or condition, or for reducing levels of, or sensitivity to, adenosine or adenosine receptors in a subject, comprising administering to a subject in need of such treatment a prophylactically or therapeutically effective amount of the pharmaceutical composition of claim 1.

Samsung Bioepis Exhibit No. 1002
Page 8371

19. The method of claim 18, wherein the disorder or condition comprises asthma, chronic obstructive pulmonary disease (COPD), cystic fibrosis (CF), dyspnea, emphysema, wheezing, pulmonary hypertension, pulmonary fibrosis, hyper-responsive airways, increased adenosine or adenosine receptor levels, adenosine hyper-sensitivity, infectious diseases, pulmonary bronchoconstriction, respiratory tract inflammation or allergies, lung surfactant or ubiquinone depletion, chronic bronchitis, bronchoconstriction, difficult breathing, impeded or obstructed lung airways, adenosine test for cardiac function, pulmonary vasoconstriction, impeded respiration, Acute Respiratory Distress Syndrome (ARDS), administration of adenosine or adenosine level increasing drugs, infantile Respiratory Distress Syndrome (infantile RDS), pain, allergic rhinitis, cancer, or chronic bronchitis.

Samsung Bioepis Exhibit No. 1002
Page 8372

Fig. 1

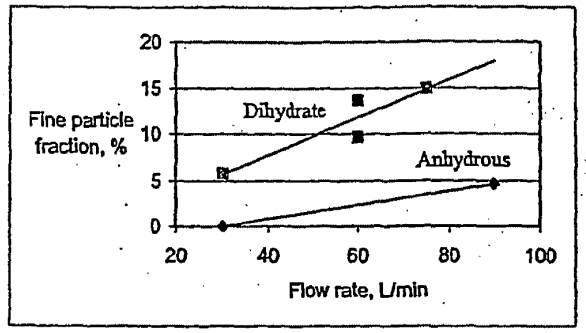
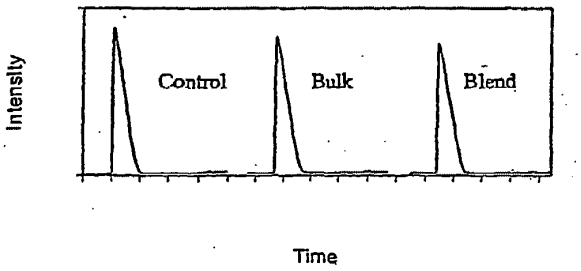


Fig. 2



Samsung Bioepis Exhibit No. 1002
Page 8373

Fig. 3

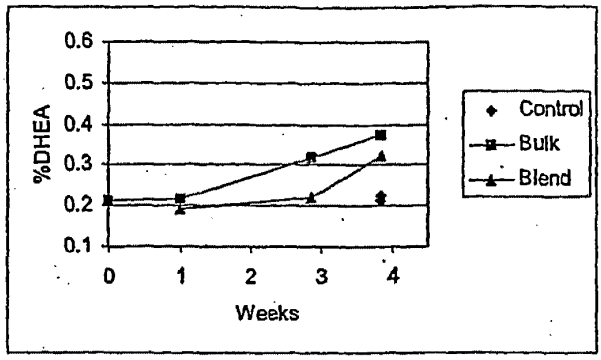
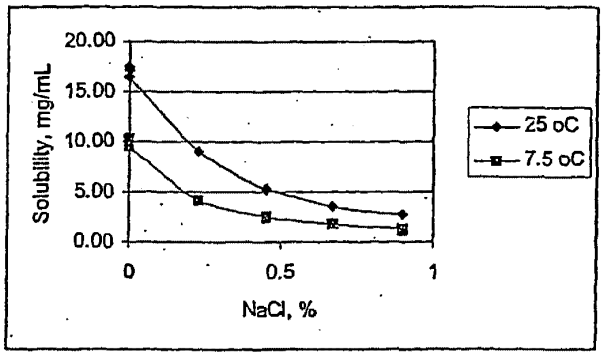


Fig. 4



Samsung Bioepis Exhibit No. 1002
Page 8374

Fig. 5

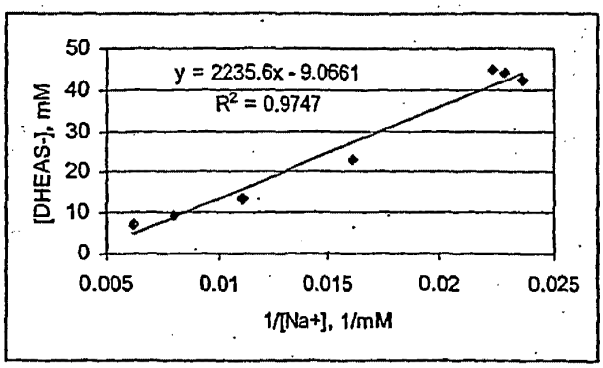
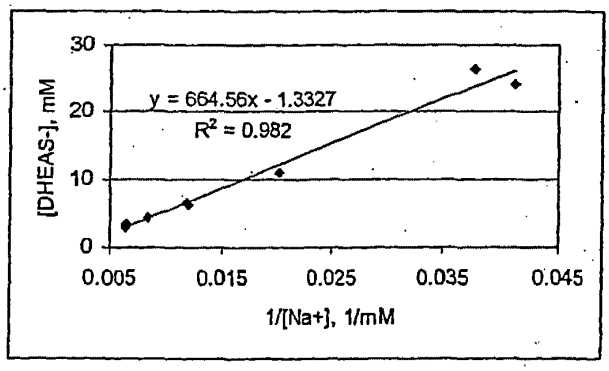


Fig. 6



Samsung Bioepis Exhibit No. 1002
Page 8375

Fig. 7

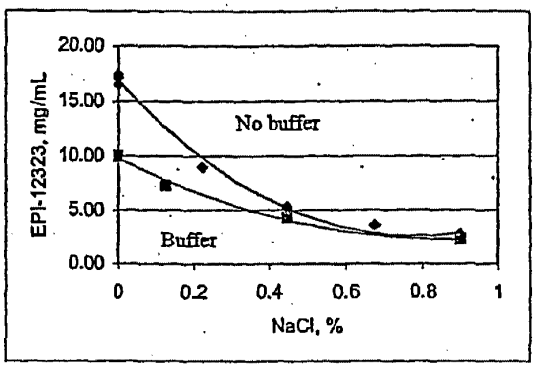
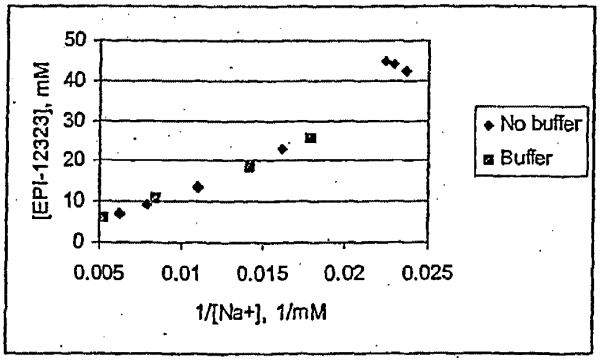


Fig. 8



Samsung Bioepis Exhibit No. 1002
Page 8376

Fig. 9

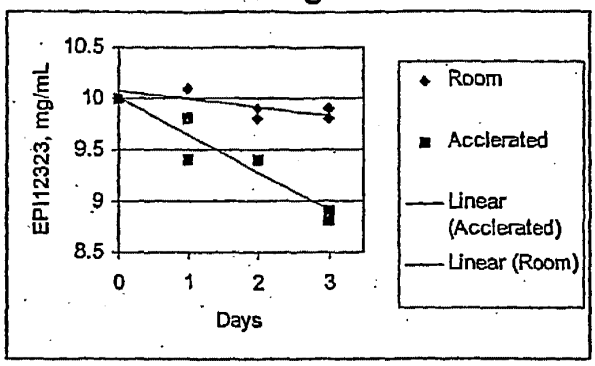
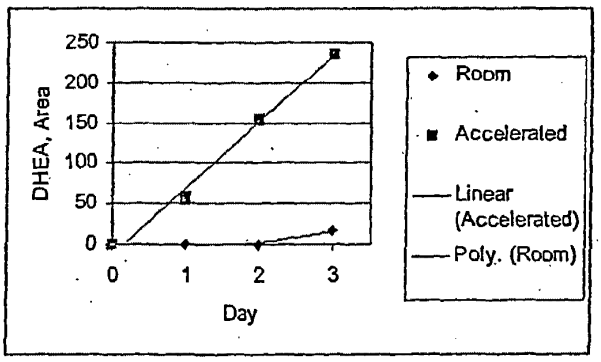
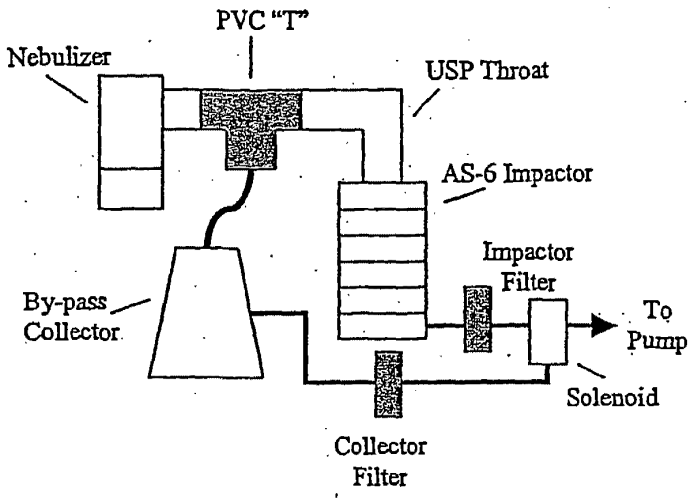


Fig. 10



Samsung Bioepis Exhibit No. 1002
Page 8377

Fig. 11



Samsung Bioepis Exhibit No. 1002
Page 8378

Fig. 12

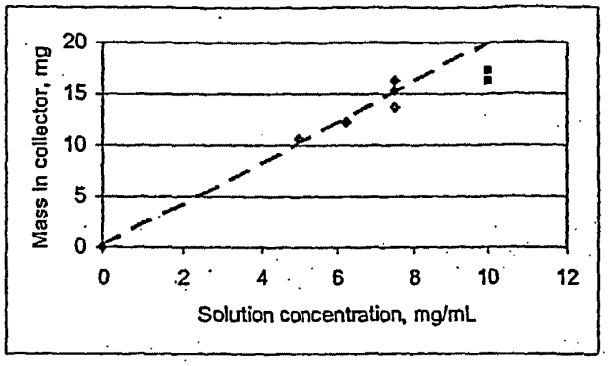
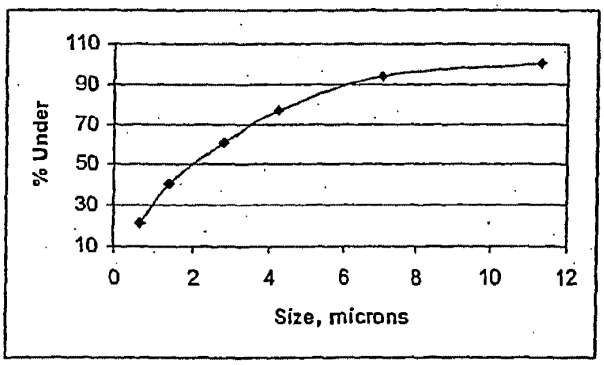
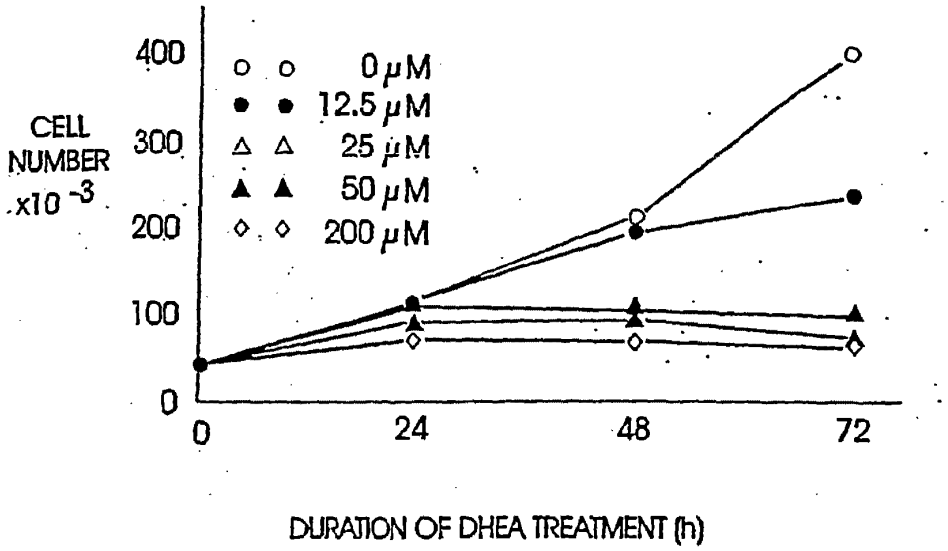


Fig. 13



Samsung Bioepis Exhibit No. 1002
Page 8379

Fig. 14



Samsung Bioepis Exhibit No. 1002
Page 8380

Fig. 15A

DURATION OF DHEA TREATMENT (h)
24
48
72
DHEA CONCENTRATION (μM)
0
25
CELL NUMBER
207
199
253
168
233
198
G1-56
S-32
G2M-12
G1-61
S-20
G2M-11
G1-70
S-21
G2M-9
G1-58
S-34
G2M-8
G1-68
S-13
G2M-19
G1-81
S-8
G2M-11
0
200
400
600
800
DNA CONTENT

250

Fig. 15B

DHEA CONCENTRATION (μM)
0
200
CELL NUMBER
211
60
G1-65
S-29
G2M-6
194
G1-68
S-16
G2M-16
265
G1-77
S-10
G2M-13
376
G1-80
S-10
G2M-10
243
G1-90
S-5
G2M-5
214
G1-90
S-5
G2M-5
0
200
400
600
800
DNA CONTENT

Fig. 16A

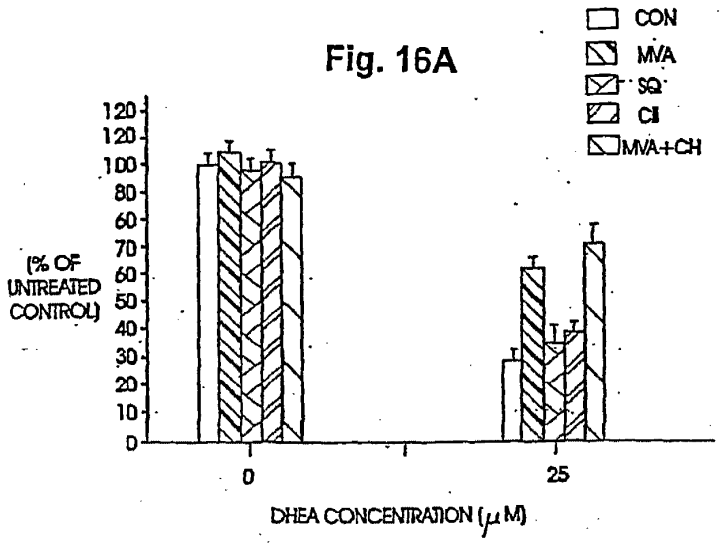
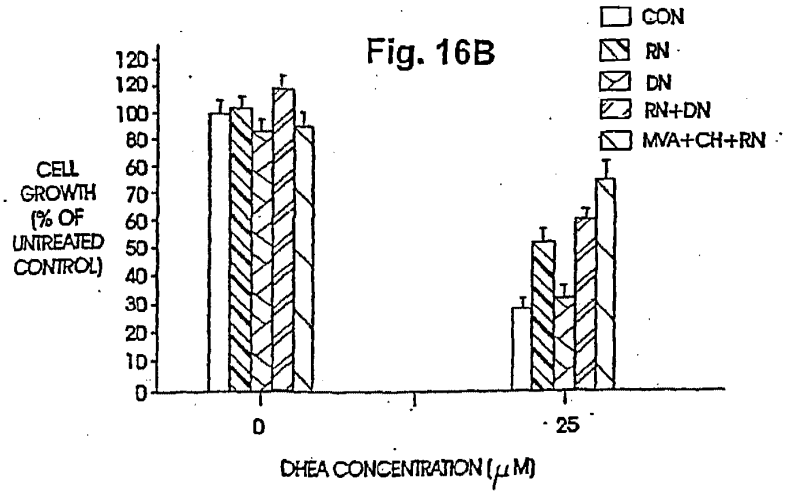
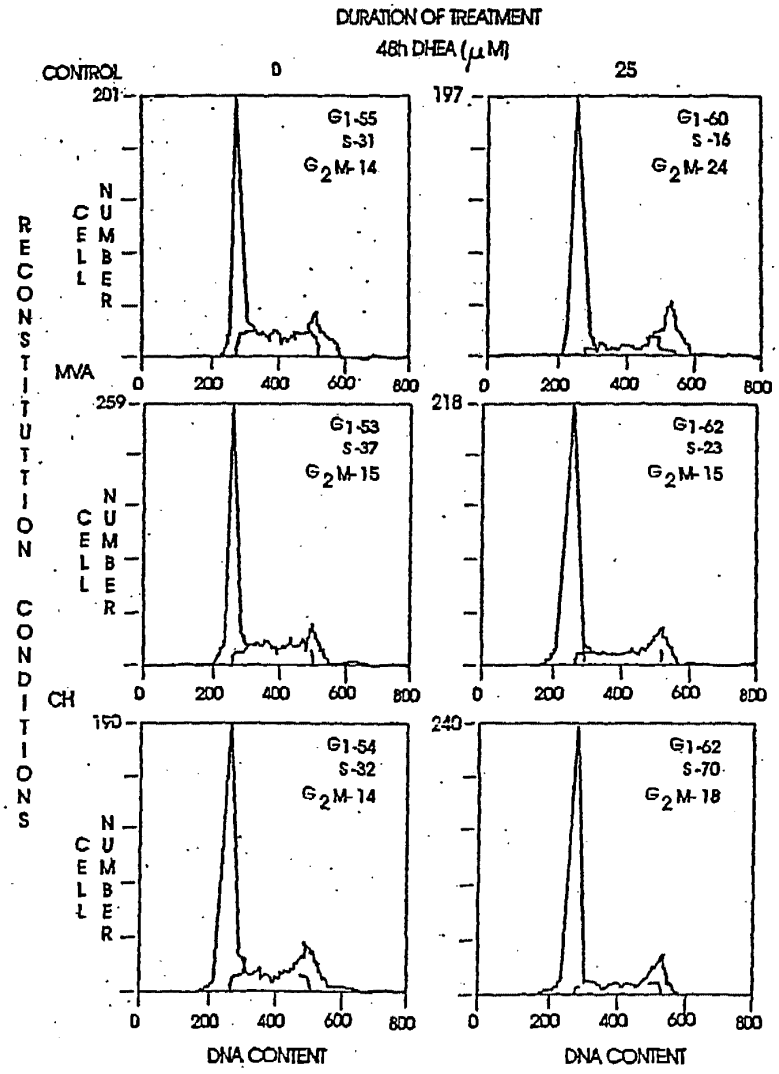


Fig. 16B



Samsung Bioepis Exhibit No. 1002
Page 8383

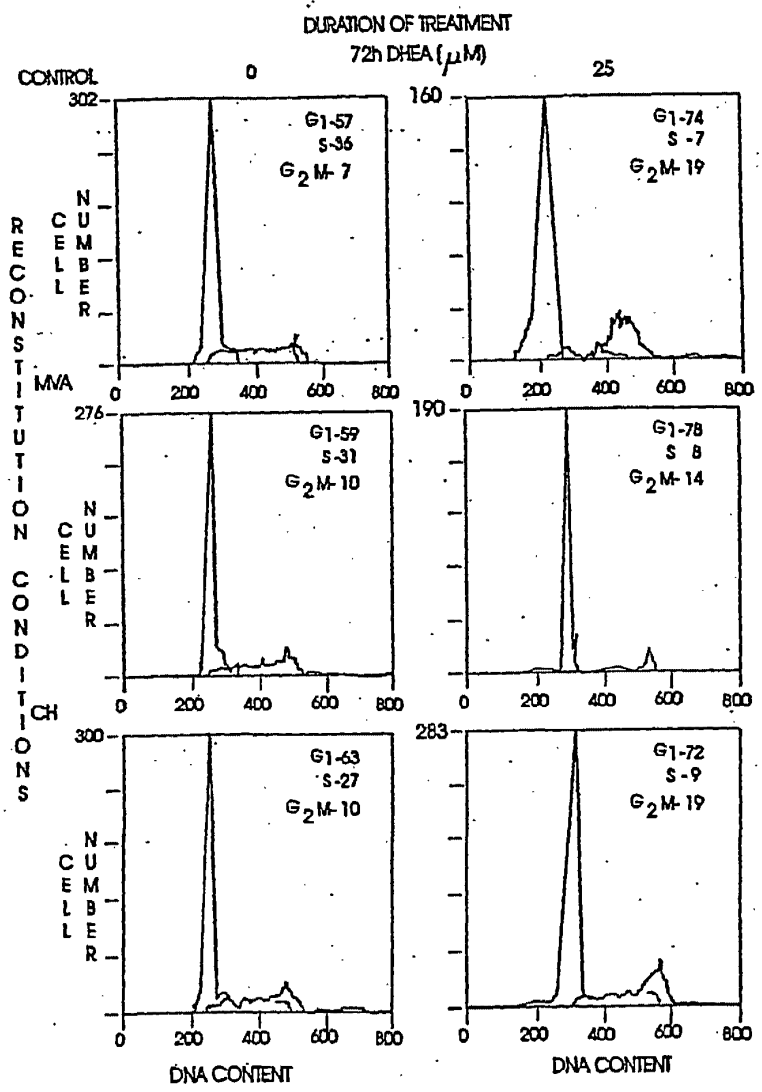
Fig. 17A



Samsung Bioepis Exhibit No. 1002
Page 8384

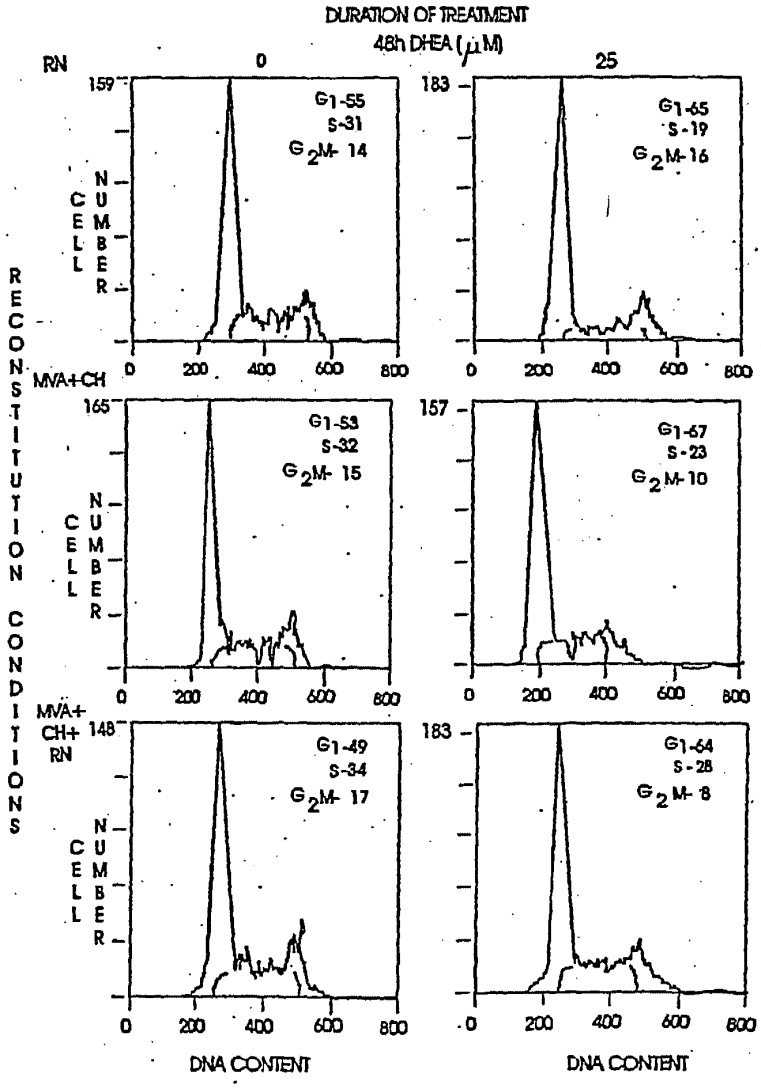
13/23

Fig. 17B



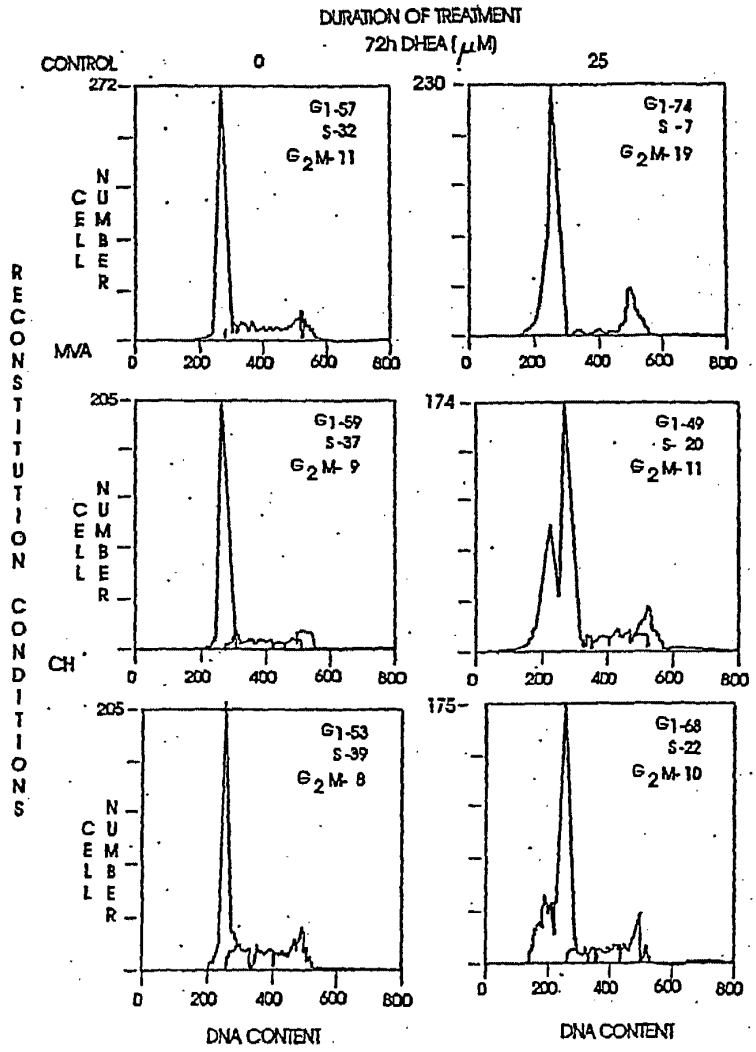
Samsung Bioepis Exhibit No. 1002
Page 8385

Fig. 17C



Samsung Bioepis Exhibit No. 1002
Page 8386

Fig. 17D



Samsung Bioepis Exhibit No. 1002
Page 8387

LIGHT CHAIN

```
                10        20        30        40
e27     DIQLTQSPSS LSASVGDRVT ITCRASKPVD GEGDSYMNWY
e26     DIQLTQSPSS LSASVGDRVT ITCRASKPVD GEGDSYMNWY
e426    DIQLTQSPSS LSASVGDRVT ITCRASQSVD YEGDSYMNWY
e25     DIQLTQSPSS LSASVGDRVT ITCRASQSVD YDGDSYMNWY
                                      CDR-L1

                50        60        70        80
e27     QQKPGKAPKL LIYAASYLES GVPSRFSGSG SGTDFTLTIS
e26     QQKPGKAPKL LIYAASYLES GVPSRFSGSG SGTDFTLTIS
e426    QQKPGKAPKL LIYAASYLES GVPSRFSGSG SGTDFTLTIS
e25     QQKPGKAPKL LIYAASYLES GVPSRFSGSG SGTDFTLTIS
                          CDR-L2

                90        100       110
e27     SLQPEDFATY YCQQSHEDPY TFGQGTKVEI KRTV
e26     SLQPEDFATY YCQQSHEDPY TFGQGTKVEI KRTV
e426    SLQPEDFATY YCQQSHEDPY TFGQGTKVEI KRTV
e25     SLQPEDFATY YCQQSHEDPY TFGQGTKVEI KRTV
                          CDR-L3
```

HEAVY CHAIN

```
                10        20        30        40
e27     EVQLVESGGG LVQPGGSLRL SCAVSGYSIT SGYSWNWIRQ
e26     EVQLVESGGG LVQPGGSLRL SCAVSGYSIT SGYSWNWIRQ
e426    EVQLVESGGG LVQPGGSLRL SCAVSGYSIT SGYSWNWIRQ
e25     EVQLVESGGG LVQPGGSLRL SCAVSGYSIT SGYSWNWIRQ
                                      CDR-H1

                50        60        70        80
e27     APGKGLEWVA SITYDGSTNY NPSVKGRITI SRDDSKNTFY
e26     APGKGLEWVA SITYDGSTNY NPSVKGRITI SRDDSKNTFY
e426    APGKGLEWVA SITYDGSTNY NPSVKGRITI SRDDSKNTFY
e25     APGKGLEWVA SITYDGSTNY NPSVKGRITI SRDDSKNTFY
                          CDR-H2

                90        100       110
e27     LQMNSLRAED TAVYYCARGS HYFGHWHFAV WGQG
e26     LQMNSLRAED TAVYYCARGS HYFGHWHFAV WGQG
e426    LQMNSLRAED TAVYYCARGS HYFGHWHFAV WGQG
e25     LQMNSLRAED TAVYYCARGS HYFGHWHFAV WGQG
                          CDR-H3
```

Fig. 18

Samsung Bioepis Exhibit No. 1002
Page 8388

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/127,438	05/11/2005	Yi Wang	ALXN-P02-102	5259

28120 7590 05/14/2008
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

EXAMINER	
VANDERVEGT, FRANCOIS P	
ART UNIT	PAPER NUMBER
1644	
MAIL DATE	DELIVERY MODE
05/14/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/127,438	WANG, YI	
	Examiner	Art Unit	
	F. Pierre VanderVegt	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 1 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☐ Responsive to communication(s) filed on _____.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-30* is/are pending in the application.

4a) Of the above claim(s) _____ is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☐ Claim(s) _____ is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☒ Claim(s) *1-30* are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☐ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date _____.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

Upon further review of the claimed inventions, the Office Action mailed October 5, 2007 is hereby vacated and replaced in its entirety with the following Election/Restriction requirement.

Election/Restrictions

1. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claim 4, 6 and 7, drawn to a method for preventing or treating a pulmonary condition using an antibody to complement component C5 or a fragment thereof, classified in class 424, subclass 133.1.

II. Claim 5, drawn to a method for preventing or treating a pulmonary condition using an antibody to C5 convertase, classified in class 424, subclass 143.1.

III. Claims 15-21, drawn to an aerosol composition comprising an antibody that inhibits activation of the complement cascade and a biopharmaceutical package comprising a nebulizer, classified in class 516, subclass 1; class 239, subclass 338 and class 530, subclass 387.1.

IV. Claims 22-30, drawn to a pulmonary drug delivery kit, classified in class 424, subclass 143.1.

Claims 1-3 and 8-14 link inventions I-II. The restriction requirement between the linked inventions is subject to the nonallowance of the linking claims, claims 1-3 and 8-14. Upon the allowance of the linking claim(s), the restriction requirement as to the linked inventions shall be withdrawn and any claim(s) depending from or otherwise including all the limitations of the allowable linking claim(s) will be entitled to examination in the instant application. Applicants are advised that if any such claim(s) depending from or including all the limitations of the allowable linking claim(s) is/are presented in a continuation or divisional application, the claims of the continuation or divisional application may be subject to provisional statutory.

Samsung Bioepis Exhibit No. 1002
Page 8391

The inventions are distinct, each from the other because of the following reasons:

2. Inventions I and II are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different designs, modes of operation, and effects (MPEP § 802.01 and § 806.06). In the instant case, the different inventions are drawn to the use of antibodies to different target proteins. Group I is drawn to antibodies that recognize a component of the complement cascade directly, while Group II is drawn to antibodies that react with an enzyme that modulates the cascade.

3. Inventions III and I & II are related as product and processes of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case the methods of Groups I and II can also be performed with antibody preparations in different forms, such as drops or injectable preparations.

4. Inventions III and IV are unrelated. Inventions are unrelated if it can be shown that they are not disclosed as capable of use together and they have different designs, modes of operation, and effects (MPEP § 802.01 and § 806.06). In the instant case, the different inventions the kit of Group IV can comprise any type of antibody for inhalation delivery, whereas the backage of Group III is restricted to an antibody that inhibits complement cascade activation.

5. Because these inventions are independent or distinct for the reasons given above and there would be a serious burden on the examiner if restriction is not required because the inventions have acquired a separate status in the art due to their recognized divergent subject matter, restriction for examination purposes as indicated is proper.

6. This application contains claims directed to the following patentably distinct species:

In Groups I and III:

Antibodies to C5.

Antibodies to C5a.

Antibodies to the C5b-9 complex or a subunit thereof.

In Group IV, Applicant must select an antibody for pulmonary delivery.

Samsung Bioepis Exhibit No. 1002
Page 8392

The species are independent or distinct because the target proteins are different and the effects of the target molecule in an immune response is different.

Applicant is required under 35 U.S.C. 121 to elect a single disclosed species for prosecution on the merits to which the claims shall be restricted if no generic claim is finally held to be allowable. Currently, claims 1-4 and 8-29 are generic.

Applicant is advised that a reply to this requirement must include an identification of the species that is elected consonant with this requirement, and a listing of all claims readable thereon, including any claims subsequently added. An argument that a claim is allowable or that all claims are generic is considered nonresponsive unless accompanied by an election.

Upon the allowance of a generic claim, applicant will be entitled to consideration of claims to additional species which depend from or otherwise require all the limitations of an allowable generic claim as provided by 37 CFR 1.141. If claims are added after the election, applicant must indicate which are readable upon the elected species. MPEP § 809.02(a).

7. Applicant is advised that the reply to this requirement to be complete must include (i) an election of a species or invention to be examined even though the requirement be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.

The election of an invention or species may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election shall be treated as an election without traverse.

Should applicant traverse on the ground that the inventions or species are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions or species to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C.103(a) of the other invention.

8. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be amended in compliance with 37 CFR 1.48(b) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. Any amendment of

Samsung Bioepis Exhibit No. 1002
Page 8393

inventorship must be accompanied by a request under 37 CFR 1.48(b) and by the fee required under 37 CFR 1.17(i).

9. Any inquiry concerning this communication or earlier communications from the examiner should be directed to F. Pierre VanderVegt whose telephone number is (571)272-0852. The examiner can normally be reached on M-Th 6:30-4:00 and Alternate Fridays 6:30-3:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Eileen O'Hara can be reached on (571) 272-0878. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

F. Pierre VanderVegt, Ph.D. /PV/
Patent Examiner
May 12, 2008

/David A Saunders/
Primary Examiner, Art Unit 1644

Samsung Bioepis Exhibit No. 1002
Page 8394

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11127438	WANG, YI
	Examiner	Art Unit
	F. Pierre VanderVegt	1644

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/12/2008								
	1	÷								
	2	÷								
	3	÷								
	4	÷								
	5	÷								
	6	÷								
	7	÷								
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OIPE IAP84
JUN 16 2008
PATENT & TRADEMARK OFFICE

06-18-08

IFW

PTO/SB/21 (01-08)
Approved for use through 04/30/2008. OMB 0651-0031
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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission	

Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F. P. Vandervegt
Attorney Docket Number	ALXN-P02-102

ENCLOSURES (*Check all that apply*)

- [ ] Fee Transmittal Form
  - [ ] Fee Attached
- [x] Amendment and Response to Restriction Requirement
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [ ] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [x] Other Enclosure(s) (please Identify below):

Return Receipt Postcard

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	ROPES & GRAY LLP		
Signature			
Printed name	Ryan Murphey, Ph.D.		
Date	June 16, 2008	Reg. No.	61,156

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EM125016052US, on the date shown below in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: June 16, 2008 Signature: Andrea Silverman (Andrea Silverman)

3628150_1.DOC

Samsung Bioepis Exhibit No. 1002
Page 8396

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EM125016052US on the date shown below in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: June 16, 2008 Signature: (Andrea Silverman)

OIPE JUN 16 2008 PATENT & TRADEMARK OFFICE

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES Vandervegt, Francois P.

AMENDMENT AND RESPONSE TO RESTRICTION REQUIREMENT

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This Response is filed in response to the Office Action mailed May 14, 2008. As June 14, 2008 is a Saturday, this response is timely filed. Please enter the following amendments:

Amendments to the Claims begin on page 2 of this paper

Remarks begin on page 5 of this paper.

AMENDMENTS TO THE CLAIMS:

Please amend claim 22. This listing of claims will replace all prior versions and listings of claims in the application:

Listing of Claims:

1. (Original) A method for preventing or treating a pulmonary condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade and an additional active agent.

2. (Original) The method of claim 1, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD) interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

3. (Original) The method of claim 1, wherein the subject is a human.

4. (Original) The method of claim 1, wherein the antibody is specific to C5.

5. (Original) The method of claim 1, wherein the antibody is specific to C5 convertase.

6. (Original) The method of claim 1, wherein the antibody is specific to C5a or C5b-9.

7. (Original) The method of claim 1, wherein the antibody is eculizumab or pexelizumab.

8. (Original) The method of claim 1, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

9. (Original) The method of claim 7, wherein the mutated Fc portion does not activate complement.

10. (Original) The method of claim 1, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.

11. (Original) The method of claim 1, wherein the additional active agent is a corticosteroid.

12. (Original) The method of claim 1, wherein the corticosteroid is dexamethasone.

13. (Original) The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by the same delivery method or route.

14. (Original) The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by different delivery methods or routes.

15. (Original) An aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for preventing or treating a pulmonary condition in a subject.

16. (Original) The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. (Original) The aerosol composition of claim 15 further comprising an additional active agent.

18. (Original) A biopharmaceutical package comprising: a) an antibody that inhibits activation of the complement cascade; and b) a nebulizer, wherein the package is suitable for preventing or treating a pulmonary condition in a subject.

19. (Original) The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. (Original) The biopharmaceutical package of claim 19 further comprising instructions for use.

21. (Original) The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. (Currently Amended) A pulmonary drug delivery kit comprising: a) an inhaler; and b) an antibody formulated for inhalation delivery, wherein said antibody inhibits activation of the complement cascade.

23. (Original) The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. (Original) The kit of claim 23, whereon the metered dose inhaler is a pressurized metered dose inhaler.

25. (Original) The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. (Original) The kit of claim 22, wherein said inhaler is a nebulizer.

27. (Original) The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

28. (Original) The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. (Original) The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. (Original) A pulmonary drug delivery kit comprising a) an anti C5 antibody composition comprising 1 mg/ml to 200 mg/ml; and b) a label indicating administration by nebulization.

Samsung Bioepis Exhibit No. 1002
Page 8400

REMARKS

Claims 1-30 are pending. Applicants have amended claim 22 to improve its form and to more particularly point out certain embodiments of Applicants' invention. Specifically, applicants have amended claim 22 to recite wherein said antibody inhibits activation of the complement cascade. Support for these amendment is found throughout the specification, for example at page 18, lines 5-27 and original claim 22. Accordingly, no new matter has been introduced.

In response to the restriction requirement set forth in the Office Action mailed May 14, 2008, applicant hereby provisionally elects Group III (claims 15-21), directed to an aerosol composition, for continued examination.

Applicants note that claims 22-30 should also be included in the examination of Group III. In the Restriction Requirement the Examiner stated that the Group III claims were drawn to an aerosol composition that inhibits activation of the complement cascade and a biopharmaceutical package comprising a nebulizer, which comprises a composition that inhibits activation of the complement cascade. All of these claims are drawn to compositions that inhibit activation of the complement cascade. Group IV, claims 22-30, is drawn to a pulmonary delivery kit, but as amended these claims are also drawn to compositions that inhibit activation of the complement cascade. In fact, claim 30 was already drawn to a kit comprising an anti-C5 antibody which inhibits activation of complement. Accordingly, we believe that the restriction is improper. Rejoinder of Groups III and IV is requested.

Applicants hereby elect "C5" as the species for search purposes only.

Applicants submit that claims 1-4 and 7-30 read on the elected species.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Applicants request that any fee required for consideration of this submission be charged to Deposit Account No. 18-1945 from which the undersigned is authorized to draw under Order No. ALXN-P02-102.

Dated: June 16, 2008

Respectfully submitted,

By _____

Ryan Murphey, Ph.D.
Registration No.: 61,156
ROPES & GRAY LLP
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Boston, Massachusetts 02110
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(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

PTO/SB/06 (07-06)
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/127,438	05/11/2005	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	06/16/2008	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 31	Minus	** 31	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	* 5	Minus	***5	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☒ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/PEGGY s. YARBOROUGH/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Samsung Bioepis Exhibit No. 1002
Page 8403

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/127,438	05/11/2005	Yi Wang	ALXN-P02-102	5259

28120 7590 09/04/2008
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

EXAMINER	
VANDERVEGT, FRANCOIS P	
ART UNIT	PAPER NUMBER
1644	

MAIL DATE	DELIVERY MODE
09/04/2008	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/127,438	WANG, YI	
	Examiner	Art Unit	
	F. Pierre VanderVegt	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *16 June 2008*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-30* is/are pending in the application.

4a) Of the above claim(s) *1-14* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *15-30* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *20060309, 20060925, 20070419, 20080225*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

Samsung Bioepis Exhibit No. 1002
Page 8406

DETAILED ACTION

This application is a continuation-in-part of U.S. Application Serial Number 10/655,861, which claims the benefit of the filing date of provisional applications 60/408,571 and 60/469189.

Claims 1-30 are currently pending.

Election/Restrictions

1. Applicant's election of Group III, claims 15-21, in the reply filed on June 16, 2008 is acknowledged. The Examiner agrees with Applicant's assertions that claim 30 should have been included with the invention of Group III. Accordingly, claim 30 is also rejoined with the invention of Group III.

It is noted that claim 22 has been amended to read upon the invention of Group III. Accordingly, claims 22-29 are rejoined with the invention of Group III.

Applicant's election of the species "C5" in the reply filed on June 16, 2008 is acknowledged. Because applicant did not distinctly and specifically point out the supposed errors in the restriction requirement regarding the species, the election has been treated as an election without traverse (MPEP § 818.03(a)).

Claims 1-14 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on June 16, 2008.

Claims 15-30 read upon the elected invention and upon the elected species. Accordingly**, claims 15-30 are the subject of examination** in the present Office Action.

Claim Objections

2. Claims 18-21 are objected to because of the following informalities: Claim 18 is ambiguous in reciting "wherein the package is suitable for preventing or treating a pulmonary condition in a subject." A package is not used to treat a subject, rather it is used to hold a medicament for that purpose.

Appropriate correction is required.

Claim Rejections - 35 USC § 112

Samsung Bioepis Exhibit No. 1002
Page 8407

The following is a quotation of the first paragraph of 35 U.S.C. 112:

> The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

3. Claims 15-21 are rejected under 35 U.S.C. 112, first paragraph, because the specification, while being enabling for treating asthma attack or treating other pulmonary conditions, does not reasonably provide enablement for preventing asthma or other pulmonary conditions. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to use the invention commensurate in scope with these claims.

Factors to be considered in determining whether undue experimentation is required are summarized in Ex parte Forman, 230 USPQ 546 (BPAI 1986). They include the nature of the invention, the state of the prior art, the relative skill of those in the art, the amount of direction or guidance disclosed in the specification, the presence or absence of working examples, the predictability or unpredictability of the art, the breadth of the claims, and the quantity of experimentation which would be required in order to practice the invention as claimed.

The claims broadly recite the prevention of pulmonary conditions. The specification defines pulmonary conditions as including a wide range of conditions, such as "asthma, bronchial constriction, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, or-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders" (page 3, lines 17-20 for example).
However, the specification shows only that treatment with the compositions of the present invention can reduce the symptoms of airway hyper-responsiveness (AHR), not completely block them. The term "preventing" is accepted to mean keeping something from happening or to anticipate or counter in advance (American Heritage College Dictionary, page 1085 in particular. However, the specification does not disclose that treatment with the compositions of the claimed invention are able to completely block AHR from occurring. Rather, the examples show that treatment is able to reduce AHR in *in vivo* models.

Given nature of the claimed invention, the breadth of the claims, the lack of guidance in the specification, the showing of working examples and the inability of one skilled in the art to predict whether the claimed method would "prevent" pulmonary conditions, it would require an undue amount of experimentation on the part of the artisan to practice the invention in a manner commensurate with the claims and this is not sanctioned by the statute.

Samsung Bioepis Exhibit No. 1002
Page 8408

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

4. Claims 15, 16, and 18-20 are rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CQ on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449) and U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892).

Drouin teaches that C5a receptors are increased on bronchial epithelial and smooth muscle cells in sepsis and in asthma (Abstract in particular). Drouin teaches that septic primates and rats that are treated with anti-C5a antibodies have reduced pulmonary edema and lung injury (page 2031, first column in particular).

Drouin does not teach a disperser for dispersing a medicament composition in an aerosol.

The '795 patent teaches a nebulizer which can be used to aerosolize medicants for nasal inhalation (Figure 4 and column 6, line 7 through column 8, line 54 in particular). The '795 patent further teaches that said nebulizer is suitable for use with viscous or sticky substances (column 8, lines 34-37 in particular). The '795 patent teaches that the nebulizer uses a jet of air to disperse the medicament (column 4, lines 35-39 in particular)[claims 18, 19]. The '795 patent also teaches the use of ultrasonic nebulizers in the art {column 1, lines 23-34 in particular)[claim 19].

As stated, the '795 patent further teaches that said nebulizer is suitable for use with viscous or sticky substances. The '734 patent specifically teaches that aqueous antibody solutions can be delivered to airways using a nebulizer (column 6, lines 36-41 and column 12, lines 37-52 in particular).

It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the nebulizer taught by the '795 patent to administer the anti-C5a antibodies taught by Drouin. One would have been motivated with a reasonable expectation of success to administer the antibodies directly to the respiratory mucosa, which is often the first line of encounter of an immune system with pathogenic organisms and by the teachings of the '795 patent that the nebulizer is usable with

Samsung Bioepis Exhibit No. 1002
Page 8409

sticky substances, which a common property of proteinaceous solutions and the '734 patent teaches the effective delivery of antibodies to pulmonary tissue using a nebulizer.

Claim 20 further asserts a limitation of comprising instructions for the use of the composition. The printed matter on the label or package insert does not lend patentable weight as a limitation of the claimed invention because the recited intended use does not physically or chemically change the nature of the agent within the container and the artisan can still use the agent for other purposes. The intended use of a product, in this case an anti-C5 antibody, does not materially change the product as the product can still be used for other purposes, such as assaying C5 binding or immunopurification of C5, which are routinely practiced in the art. The recited "limitations" only further limit the intended use method printed on the label or package insert and do not change the physical and/or chemical properties of the components of the kit. In *In re* Haller (73 USPQ 403 (CCPA 1947)) the Court decided upon a case in which "it is not alleged by the Examiner or the board that the use of the involved compound as an insecticide is old or obvious and, in fact a claim covering broadly a process comprising the application of that compound to insects for the purpose of destroying them, has been allowed. In the instant case the appealed claim calls for a packaged product of an old compound labeled to indicate that the compound is intended for use as an insecticide." It is respectfully submitted that the facts of In re Haller are directly pertinent to the instant case. The method of treating an individual using compounds and compositions such as those contained in the instantly claimed kit has already been allowed in a parent application. Applicant relies therefore, as in In re Haller, upon printed matter reciting an intended use of the otherwise old compounds and compositions. The Court noted in *In re* Haller that "It is well settled that the application of particular printed matter to an old article cannot render the article patentable" and decided that, "the mere labeling of an old composition as an insecticide does not make it a new or different composition within the meaning of the patent statutes" and "Whether the statement of intended use appears merely in the claim or in a label on the product is immaterial so far as the question of patentability is concerned" in affirming the rejection. In *In re* Venezia (189 USPQ 149 (CCPA 1976)) the Court decided that the kit components were limited by the instructions listed in the claims because said kit components had to be adapted in a particular manner in order to meet the final assembly instructions that were part of the claim. The Court stated however that, "what may or may not happen in the future is not a part of the claimed invention" (emphasis by Court). The Court decided that the instructions of the claim were material to the claim because, "Rather than being a mere direction of activities to take place in the future, this language imparts a structural limitation to the sleeve" (emphasis added). The court reasoned this position by stating, "this language also defines present structures or attributes of the part of the 'kit'

Samsung Bioepis Exhibit No. 1002
Page 8410

identified as the housing, which limits the structure of the housing to those configurations which allow for the completed connector assembly desired." It is respectfully submitted that, in the instant case, the instructions do not modify the reagent comprised in the claimed kit, i.e., the reagent does not have to be altered for use commensurate with the instructions, rather the printed instructions merely set forth steps for what may be done with the kit components in the future. Accordingly, the printed instructions do not lend patentable weight to the claimed invention.

5. Claims 17 and 21 are rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CP on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449) and U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892) as applied to claims 15 and 18 above, and further in view of U.S. Patent No. 5,728,844 to Muller et al. (D on form PTO-892).

Drouin, the '795 patent and the '734 patent have been discussed supra.

The combined references do not specifically teach an additional active agent.

The ‘844 patent teaches that it is common in the art to treat sepsis patients with dexamethasone in order to counter the effects of TNF.alpha. and that treatment with dexamethasone can be combined with the use of other medicaments (column 4, line 66 to column 5, line 20 in particular).

It would have been prima facie obvious to a person having ordinary skill in the art at the time the invention was made to add dexamethasone as an active agent in a treatment composition as taught by the combined teachings of Drouin, the '795 patent and the '734 patent. One would have been motivated to make the combination with a reasonable expectation of success by the teachings of the ‘844 patent that it is desirable to inhibit TNF.alpha. during the treatment of sepsis and that use of dexamethasone can be combined with the use of other medicaments.

6. Claims 22, and 25-30 are rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CP on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449) and U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892) as applied to claims 15 and 18 above, and further in view of U.S. Patent No. 6,524,836 to Sheppard (B on form PTO-892).

Drouin, the '795 and the '734 patent have been discussed supra.

The combined references do not specifically teach a kit or dry powder inhaler.

Samsung Bioepis Exhibit No. 1002
Page 8411

The ‘836 patent teaches kits comprising a nebulizer or dry powder inhaler for the delivery of medicaments to a subject, including medicaments comprising antibodies as an active agent (column 56, lines 48-62 in particular).

It would have been prima facie obvious to a person having ordinary skill in the art at the time the invention was made to package the active composition as taught by the combination of Drouin, the '795 and the '734 patent in a kit form as taught by the ‘836 patent.. One would have been motivated to assemble such a kit to make it easier for a practitioner or patient to store and use the components needed for treatment. One would have been further motivated to use a dry powder instead of a liquid suspension for similar reasons of storage and ease of use.

Claim 30 is included because, while the cited references do not specifically teach a concentration of anti-C5 antibodies to use in a pulmonary delivery composition, it would be routine well within the purview of one of ordinary skill in the art to determine such a concentration.

7. Claims 23 and 24 are rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CP on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449), U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892) and U.S. Patent No. 6,524,836 to Sheppard (B on form PTO-892) as applied to claim 22 above, and further in view of U.S. Patent No. 6,905, 688 to Rosen et al. (C on form PTO-892).

Drouin, the '795 and the '734 patent have been discussed supra.

The combined references do not specifically teach a metered dose inhaler.

The ‘688 patent teaches the use of a metered dose inhaler as an alternative to the use of a nebulizer or dry powder inhaler for the aerosol delivery of a large protein medicament to a subject (column 17, lines 19-34 in particular). The ‘688 patent teaches that the composition can be supplied in the form of a kit (column 2, lines 51-61 in particular).

It would have been prima facie obvious to a person having ordinary skill in the art at the time the invention was made to use a metered dose inhaler as taught by the ‘688 patent in combination with the teachings of Drouin, the '795 and the '734 patent. One would have been motivated to use a metered dose inhaler as taught by the '688 patent with a reasonable expectation of success by the teachings of the ‘688 patent showing that a metered dose inhaler is an alternative to nebulizers and dry powder inhalers for the delivery of a medicament comprising a large protein active agent and perhaps by the need to have an exact measurement of the dose delivered to a subject, which may not be possible with a nebulizer or dry powder inhaler.

Conclusion

8. No claim is allowed.

9. Any inquiry concerning this communication or earlier communications from the examiner should be directed to F. Pierre VanderVegt whose telephone number is (571)272-0852. The examiner can normally be reached on M-Th 6:30-4:00 and Alternate Fridays 6:30-3:00.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Eileen O'Hara can be reached on (571) 272-0878. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

F. Pierre VanderVegt, Ph.D. /PV/
Patent Examiner
August 26, 2008

/Eileen B. O'Hara/
Supervisory Patent Examiner
Art Unit 1644

Samsung Bioepis Exhibit No. 1002
Page 8413

Notice of References Cited	Application/Control No. 11/127,438	Applicant(s)/Patent Under Reexamination WANG, YI	
	Examiner F. Pierre VanderVegt	Art Unit 1644	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-5,871,734	02-1999	Lobb et al.	424/144.1
*	B	US-6,524,836	02-2003	Sheppard, Paul O.	435/226
*	C	US-6,905,688	06-2005	Rosen et al.	424/192.1
*	D	US-5,728,844	03-1998	Muller et al.	548/472
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	American Heritage College Dictionary, 3rd Edition. [1997]. Houghton Mifflin Company, Boston. page 1085.
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11127438	WANG, YI
	Examiner	Art Unit
	F. Pierre VanderVegt	1644

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
PALM Inventor, 10/655,861, 60/408,571, 60/469,189 EAST, STN Medline Embase scisearch	8/26/08	PV
search strategy ATTACHED	8/28/08	PV

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

EAST Search History

Ref #	Hits	Search Query	DBs	Default Operator	Plurals	Time Stamp
L1	3092	nebulizer same insufflator	US-PGPUB; USPAT; DERWENT	OR	OFF	2008/08/28 08:03
L2	8	nebulizer same insufflator same antibody	US-PGPUB; USPAT; DERWENT	OR	OFF	2008/08/28 08:04
L5	214	nebulizer same antibody same dry ADJ powder	US-PGPUB; USPAT; DERWENT	OR	OFF	2008/08/28 08:08
L6	5364	metered dose inhaler	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 08:50
L7	1905	metered dose inhaler same nebulizer	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 08:50
L8	997	metered dose inhaler same nebulizer same dry powder	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 08:50
L9	0	metered dose inhaler same nebulizer same dry powder smae antibody	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 08:51
L10	0	metered dose inhaler same nebulizer same dry powder smae protein	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 08:51
L11	87	metered dose inhaler same nebulizer same dry powder same protein	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 08:51
L12	186	sepsis same dexamethasone	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 09:58
L13	144	sepsis same dexamethasone and pulmonary	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 09:58
L14	28	sepsis same dexamethasone same pulmonary	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 09:59
L15	2296	pulmonary and complement and dexamethasone	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 10:09
L16	0	pulmonary same complement samedexamethasone	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 10:09
L17	3	pulmonary same complement same dexamethasone	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 10:09

Samsung Bioepis Exhibit No. 1002
Page 8416

L18	56	dexamethasone same treatment same sepsis	US-PGPUB; USPAT; DERWENT	ADJ	OFF	2008/08/28 10:13

8/28/2008 10:52:41 AM
C:\Documents and Settings\FVandervegt\My Documents\EAST\\default.wsp

Samsung Bioepis Exhibit No. 1002
Page 8417

OIPE IAP43 MAR 09 2006 PATENT & TRADEMARK OFFICE

I hereby certify that this correspondence is being deposited with the U.S. Postal Service with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: March 6, 2006 Signature: (Mary Jane DiPalma)

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Wang Yi

Application No.: 11/127438

Confirmation No.: 5259

Filed: May 11, 2005

Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: Vandervegt, Francois P.

INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

Applicant has not submitted copies of each cited U.S. patent and U.S. patent application as required by 37 CFR 1.98(a)(2)(i), amended October 2004, as the U.S. Patent and Trademark Office has waived this requirement for all U.S. patent applications. Applicant submits herewith copies of foreign and non-patents in accordance with 37 CFR 1.98(a)(2). Also enclosed is a copy of a Search Report from a corresponding European application.

Those documents which are marked with a double asterisk (**) next to the Cite No. in the attached form PTO/SB/08 are not supplied because they were previously cited by or

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Samsung Bioepis Exhibit No. 1002
Page 8418

submitted to the Office in a prior application number 10/655861, filed September 5, 2003 and relied upon in this application for an earlier filing date under 35 U.S.C. 120.

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: March 6, 2006

Respectfully submitted,

By_____

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Samsung Bioepis Exhibit No. 1002
Page 8419

OIPE IAP43 MAR 09 2006 PATENT & TRADEMARK OFFICE

PTO/SB/08a/b (07-05)
Approved for use through 07/31/2006. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it contains a valid OMB control number.

Substitute for form 1449A/B/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete If Known	
Application Number	11/127438
Filing Date	May 11, 2005
First Named Inventor	Wang Yi
Art Unit	1644
Examiner Name	Vandervegt, Francois P.
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
	AA**	US-6,316,502	11-13-2001	Lai et al.	
	AB**	US-6,355,245	03-12-2002	Evans et al.	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
	BA**	EP-0649468-B1	03-15-2000	AdProTech plc		

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. * CITE NO.: Those application(s) which are marked with an single asterisk (*) next to the Cite No. are not supplied (under 37 CFR 1.98(a)(2)(iii)) because that application was filed after June 30, 2003 or is available in the IFW. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CA	Abe et al., "Contribution of Anaphylatoxin C5a to Late Airway Responses after Repeated Exposure of Antigen to Allergic Rats," The Journal of Immunology 167(8):4651-4660 (2001)	
	CB	Krug et al., "Complement Factors C3a and C5a are Increased in Bronchoalveolar Lavage Fluid after Segmental Allergen Provocation in Subjects with Asthma," Am. J. Respir. Crit. Care Med. 164:1841-1843 (2001)	
	CC**	Lukacs et al., "Complement-dependent Immune Complex-induced Bronchial Inflammation and Hyperreactivity," Am. J. Physiol. Lunch Cell Mol. Physiol. 280:L512-L518 (2001)	
	CD	Peng et al., "Role of C5 in the Development of Airway Inflammation, Airway Hyperresponsiveness, and Ongoing Airway Response," The Journal of Clinical Investigation 115(6):1590-1600 (2005)	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature	/F Pierre Vandervegt/	Date Considered	08/27/2008

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Samsung Bioepis Exhibit No. 1002
Page 8420

FILE 'MEDLINE, SCISEARCH, EMBASE' ENTERED AT 07:32:33 ON 28 AUG 2008

L1 47786 S PULMONARY AND (SEPSIS OR ASTHMA)
L2 8373 S COMPLEMENT AND C5
L3 50 S L1 AND L2
L4 34 DUP REM L3 (16 DUPLICATES REMOVED)
L5 156306 S DEXAMETHASONE
L6 583 S L5 AND L1
L7 413 DUP REM L6 (170 DUPLICATES REMOVED)
L8 248 S L7 NOT PY>2003
L9 226 S L7 NOT PY>2002
L10 413 S L7 AND L6
L11 1 S L7 AND L4

Samsung Bioepis Exhibit No. 1002
Page 8421

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service on the date shown below with sufficient postage as First Class Mail, in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: September 22, 2006 Signature: (Mary Jane DiPalma)

IFW

Docket No.: ALXN-P02-102
(PATENT)

OIPE IAP80 SEP 25 2006 PATENT & TRADEMARK OFFICE

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES Examiner: Vandervegt, Francois P.

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

Applicant submits herewith copies of non-patent literature in accordance with 37 CFR 1.98(a)(2).

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ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /PV/

Samsung Bioepis Exhibit No. 1002
Page 8422

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: September 22, 2006

Respectfully submitted,

By _____

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Attorneys/Agents For Applicant

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292

OIPE IAP80
SEP 25 2006
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PTO/SB/08a/b (07-06)
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Substitute for form 1449A/B/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 3

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	Vandervegt, Francois P.
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CE	Abrahamsen et al., "Differential Mediator Release from Basophils of Allergic and Non-allergic Asthmatic Patients after Stimulation with Anti-IgE and C5a," Clinical and Experimental Allergy 31:368-378 (2001)	
	CF	Akatsu et al., "Distribution of Rat C5a Anaphylatoxin Receptor," Microbiol. Immunol. 46(12):863-874 (2002)	
	CG	Bjornson et al., "Complement is Activated in the Upper Respiratory Tract During Influenza Virus Infection," Am. Rev. Respir. Dis. 143:1062-1066 (1991)	
	CH	Blease et al., "Chemokines and Their Role in Airway Hyper-reactivity," Respir. Res. 1:54-61 (2000)	
	CI	Boulet et al., "Airway Hyperresponsiveness, Inflammation, and Subepithelial Collagen Deposition in Recently Diagnosed vs. Long-standing Mild Asthma," American Journal of Respiratory and Critical Care Medicine 162:1308-1313 (2000)	
	CJ	Chakir et al., "Airway Remodeling-associated Mediators in Moderate to Severe Asthma: Effect of Steroids on TGF-β, IL-11, IL-17, and Type I and Type III Collagen Expression," J. Allergy Clin. Immunol. 111:1293-1298 (2003)	
	CK	Chenoweth et al., "Demonstration of Specific C5a Receptor on Intact Human Polymorphonuclear Leukocytes," Proc. Natl. Acad. Sci. USA 75(8):3943-3947 (1978)	
	CL	Chung, "Cytokines in Chronic Obstructive Pulmonary Disease," Eur. Respir. J. 18(34):50s-59s (2001)	
	CM	Cieslewicz et al., "The Late, but not Early, Asthmatic Response is Dependent on IL-5 and Correlates with Eosinophil Infiltration," J. Clin. Invest. 104:301-308 (1999)	
	CN	Collard et al., "Complement Activation Following Oxidative Stress," Molecular Immunology 36:941-948 (1999)	
	CO	Czermak et al., "Complement, Cytokines, and Adhesion Molecule Expression in Inflammatory Reactions," Proceedings of the Association of American Physicians 110(5):306-312 (1998)	
	CP	Desai et al., "Demonstration of C5 Cleaving Activity in Bronchoalveolar Fluids and Cells: A Mechanism of Acute and Chronic Alveolitis," Journal of Experimental Pathology 1(3):201-216 (1984)	

Examiner Signature		Date Considered	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /PV/

Samsung Bioepis Exhibit No. 1002
Page 8424

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Substitute for form 1449A/B/PTO				Complete if Known	
INFORMATION DISCLOSURE STATEMENT BY APPLICANT				Application Number	11/127,438
				Filing Date	May 11, 2005
				First Named Inventor	Yi Wang
				Art Unit	1644
(Use as many sheets as necessary)				Examiner Name	Vandervegt, Francois P.
Sheet	2	of	3	Attorney Docket Number	ALXN-P02-102

	CQ	Drouin et al, "Expression of the Complement Anaphylatoxin C3a and C5a Receptors on Bronchial Epithelial and Smooth Muscle Cells in Models of Sepsis and Asthma," The Journal of Immunology 166:2025-2032 (2001)	
	CR	Frank, "Complement: A Brief Review," J. Allergy Clin. Immunol. 84(4,1):411-420 (1989)	
	CS	Gerard et al., "Complement in Allergy and Asthma," Current Opinion in Immunology 14:705-708 (2002)	
	CT	Glovsky et al., "Is Complement Activation a Factor in Bronchial Asthma?," Int. Arch. Allergy Immunol. 118:330-332 (1999)	
	CU	Gonczi et al., "The Severity of Clinical Symptoms in Ragweed-allergic Patients is Related to the Extent of Ragweed-induced Complement Activation in their Sera," Allergy 51:1110-1114 (1997)	
	CV	Hawlisch et al., "The Anaphylatoxins Bridge Innate and Adaptive Immune Responses in Allergic Asthma," Molecular Immunology 41:123-131 (2004)	
	CW	Hogaboam et al., "Mannose-binding Lectin Deficiency Alters the Development of Fungal Asthma: Effects on Airway Response, Inflammation, and Cytokine Profile," Journal of Leukocyte Biology 75:805-814 (2004)	
	CX	Holgate et al., "The Bronchial Epithelium as a Key Regulator of Airway Inflammation and Remodelling in Asthma," Clinical and Experimental Allergy 29:90-95 (1999)	
	CY	Höpken et al., "TheC5a Chemoattractant Receptor Mediates Mucosal Defence to Infection," Nature 383:86-89 (1996)	
	CZ	Irvin et al., "Airways Hyperreactivity and Inflammation Produced by Aerosolization of Human C5A des arg^{1-3}," Am. Rev. Respir. Dis. 134:777-783 (1986)	
	CA1	Jagels et al., "C3a and C5a Enhance Granulocyte Adhesion to Endothelial and Epithelial Cell Monolayers: Epithelial and Endothelial Priming is Required for C3a-induced Eosinophil Adhesion," Immunopharmacology 46:209-222 (2000)	
	CB1	Karp et al., "Identification of Complement Factor 5 as a Susceptibility Locus for Experimental Allergic Asthma," Nature Immunology 1(3):221-226 (2000)	
	CC1	Kodani et al., "Intratracheal Administration of Anaphylatoxin C5a Potentiates Antigen-induced Pulmonary Reactions Through the Prolonged Production of Cysteinyl-leukotrienes," Immunopharmacology 49:263-274 (2000)	
	CD1	Larsen et al., "A Differential Effect of C5a and C5a des Arg in the Induction of Pulmonary Inflammation," Am. J. Pathol. 100:179-192 (1980)	
	CE1	Maruo et al., "Generation of Anaphylatoxins Through Proteolytic Processing of C3 and C5 by House dust Mite Protease," J. Allergy Clin. Immunol. 100:253-260 (1997)	
	CF1	Mattos et al., "Matrix Metalloproteinase-9 Expression in Asthma," Chest 122:1543-1552 (2002)	
	CG1	Nagata et al., "Activation of Human Serum Complement with Allergens," J. Allergy Clin. Immunol.80:24-32 (1987)	
	CH1	Nagy et al., "The Development of Asthma in Children Infected with *Chlamydia pneumoniae* is Dependent on the Modifying Effect of Mannose-binding Lectin," J. Allergy Clin. Immunol. 112(4):729-734 (2003)	
	CI1	O'Byrne et al., "Reassessing the Th2 Cytokine Basis of Asthma," Trends in Pharmacological Sciences 25(5):244-248 (2004)	
	CJ1	Robbins et al., "Complement Activation by Cigarette Smoke," L254-L259 (1990)	
	CK1	Taube et al., "Inhibition of Complement Activation Decreases Airway Inflammation and Hyperresponsiveness," Am. J. Respir. Crit. Car Med. 168:1333-1341 (2003)	
	CL1	Teran et al., "Identification of Neutrophil Chemotactic Factors in Bronchoalveolar Lavage Fluid of Asthmatic Patients," Clinical and Experimental Allergy 27:396-405 (1997)	
	CM1	Varsano et al., "Generation of Complement C3 and Expression of Cell Membrane Complement Inhibitory Proteins by Human Bronchial Epithelium Cell Line," Thorax 55:364-369 (2000)	

Examiner Signature		Date Considered	

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /PV/

Samsung Bioepis Exhibit No. 1002
Page 8425

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 3 of 3

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	Vandervegt, Francois P.
Attorney Docket Number	ALXN-P02-102

	CN1	Wang et al., "Amelioration of Lupus-like Autoimmune Disease in NZB/WF$_1$ Mice after Treatment with a Blocking Monoclonal Antibody Specific for Complement Component C5," Proc. Natl. Acad. Sci. USA 93:8563-8568 (1996)	
	CO1	Wang et al., "Anti-C5 Monoclonal Antibody Therapy Prevents Collagen-induced Arthritis and Ameliorates Established Disease," Proc. Natl. Acad. Sci. USA 92:8955-8959 (1995)	
	CP1	Wills-Karp et al., "Interleukin-13: Central Mediator of Allergic Asthma," Science 282:2258-2261 (1998)	

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[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

Examiner Signature	/F Pierre Vandervegt/	Date Considered	08/27/2008

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Samsung Bioepis Exhibit No. 1002
Page 8426

EFW

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Dated: 4-13-07 Signature: Maura A. Gallagher (Maura B. Gallagher)
~~Elaine Leahy~~

OIPE APR 19 2007 PATENT & TRADEMARK OFFICE

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438

Confirmation No.: 5259

Filed: May 11, 2005

Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: F.P. Vandervegt

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action on the merits as far as is known to the undersigned (37 CFR 1.97(b)(3)).

In accordance with 37 CFR 1.98(a)(2)(ii), Applicant has not submitted copies of U.S. patents and U.S. patent applications. Applicant submits herewith copies of foreign patents and non-patent literature in accordance with 37 CFR 1.98(a)(2).

10489597_1

Samsung Bioepis Exhibit No. 1002
Page 8427

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: 4/13/07

Respectfully submitted,

By Anita Varma

Anita Varma, Esq.
Registration No.: 43,221
FISH & NEAVE IP GROUP, ROPES & GRAY LLP
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Attorneys/Agents For Applicant

Samsung Bioepis Exhibit No. 1002
Page 8428

OIPE IAP39 APR 19 2007 PATENT & TRADEMARK OFFICE

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet	1	of	1

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F.P. Vandervegt
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
/PV/	AC	US-4,228,795	10/21/1980	Babington	

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (*if known*)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]

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/PV/	CQ1	Fitch et al., "Pharmacology and Biological Efficacy of a Recombinant, Humanized, Single-Chain Antibody C5 Complement Inhibitor in Patients Undergoing Coronary Artery Bypass Graft Surgery with Cardiopulmonary Bypass," *Circulation* 100:2499-2506 (1999).	

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Examiner Signature	/F Pierre Vandervegt/	Date Considered	08/27/2008

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Page 8429

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(Based on PTO 01-08 version)

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F. P. Vandervegt
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
/PV/	BB	WO2004/022096	03-18-2004	Alexion Pharmaceuticals, Inc.		
/PV/	BC	WO2005/011614	02-10-2005	Epigenesis Pharmaceuticals, Inc.		

Examiner Signature		Date Considered	

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/PV/	CR1	PENG et al., 2004, Contribution of complement component C5 in the development of airway inflammation, maintaining airway hyperresponsiveness and sustaining an ongoing asthmatic attack, Abstracts/Mol. Immunol. 41:292	
/PV/	CS1	PENG et al., 2006, Blocking intrapulmonary activation of complement cascade on the development of airway hyperresponsiveness: utility in sight? J. of Allergy and Clin. Immunol. 117(3):720	

Examiner Signature	/F Pierre Vandervegt/	Date Considered	08/27/2008

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BIB DATA SHEET

CONFIRMATION NO. 5259

SERIAL NUMBER	FILING or 371(c) DATE	CLASS	GROUP ART UNIT	ATTORNEY DOCKET NO.
11/127,438	05/11/2005 RULE	514	1644	ALXN-P02-102

APPLICANTS
Yi Wang, Orange, CT;

**** CONTINUING DATA ***************************
This application is a CIP of 10/655,861 09/05/2003
which claims benefit of 60/408,571 09/06/2002
and claims benefit of 60/469,189 05/09/2003

**** FOREIGN APPLICATIONS ***************************

**** IF REQUIRED, FOREIGN FILING LICENSE GRANTED **** ** SMALL ENTITY **
09/01/2005

Foreign Priority claimed	35 USC 119(a-d) conditions met	Verified and Acknowledged	Met after Allowance	STATE OR COUNTRY	SHEETS DRAWINGS	TOTAL CLAIMS	INDEPENDENT CLAIMS
☐ Yes ☑ No	☐ Yes ☑ No	/PIERRE VANDERVEGT/ Examiner's Signature	☐ Initials	CT	16	30	5

ADDRESS

ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624
UNITED STATES

TITLE

Nebulization of monoclonal antibodies for treating pulmonary diseases

FILING FEE RECEIVED	FEES: Authority has been given in Paper	
1220	No._____ to charge/credit DEPOSIT ACCOUNT No._____ for following:	☐ All Fees ☐ 1.16 Fees (Filing) ☐ 1.17 Fees (Processing Ext. of time) ☐ 1.18 Fees (Issue) ☐ Other _____ ☐ Credit

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11127438	WANG, YI
	Examiner	Art Unit
	F. Pierre VanderVegt	1644

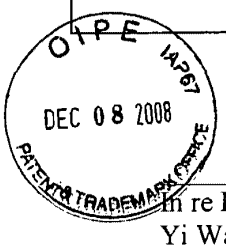
✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/12/2008	08/28/2008							
	1	÷	N							
	2	÷	N							
	3	÷	N							
	4	÷	N							
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	11	÷	N							
	12	÷	N							
	13	÷	N							
	14	÷	N							
	15	÷	✓							
	16	÷	✓							
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	28	÷	✓							
	29	÷	✓							
	30	÷	✓							

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Dated: December 8, 2008 Signature: Pamela Harrison (Pamela A. Harrison)



Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438

Confirmation No.: 5259

Filed: May 11, 2005

Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: Vandervegt, Francois P.

AMENDMENT AND RESPONSE TO OFFICE ACTION

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

This Reply is filed in response to the Office Action mailed September 4, 2008 in the above-identified application. An Information Disclosure Statement, a Request for a one-month extension of time and appropriate fees are filed concurrently herewith. Reconsideration and further examination are requested.

Listing of the Claims begins on page 2 of this paper.

Remarks/Arguments begin on page 5 of this paper.

AMENDMENTS TO THE CLAIMS

Please amend claims 15 and 18. This listing of claims will replace all prior versions and listings of claims in the application:

1. (Withdrawn) A method for preventing or treating a pulmonary condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade and an additional active agent.

2. (Withdrawn) The method of claim 1, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD) interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

3. (Withdrawn) The method of claim 1, wherein the subject is a human.

4. (Withdrawn) The method of claim 1, wherein the antibody is specific to C5.

5. (Withdrawn) The method of claim 1, wherein the antibody is specific to C5 convertase.

6. (Withdrawn) The method of claim 1, wherein the antibody is specific to C5a or C5b-9.

7. (Withdrawn) The method of claim 1, wherein the antibody is eculizumab or pexelizumab.

8. (Withdrawn) The method of claim 1, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

9. (Withdrawn) The method of claim 7, wherein the mutated Fc portion does not activate complement.

10. (Withdrawn) The method of claim 1, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.

11. (Withdrawn) The method of claim 1, wherein the additional active agent is a corticosteroid.

12. (Withdrawn) The method of claim 1, wherein the corticosteroid is dexamethasone.

13. (Withdrawn) The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by the same delivery method or route.

14. (Withdrawn) The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by different delivery methods or routes.

15. (Currently Amended) An aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for ~~preventing or~~ treating a pulmonary condition in a subject.

16. (Original) The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. (Original) The aerosol composition of claim 15 further comprising an additional active agent.

18. (Currently Amended) A biopharmaceutical package comprising: a) an antibody that inhibits activation of the complement cascade; and b) a nebulizer, wherein the antibody ~~package~~ is suitable for ~~preventing or~~ treating a pulmonary condition in a subject.

19. (Original) The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. (Original) The biopharmaceutical package of claim 19 further comprising instructions for use.

21. (Original) The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. (PreviouslyPresented) A pulmonary drug delivery kit comprising: a) an inhaler; and b) an antibody formulated for inhalation delivery, wherein said antibody inhibits activation of the complement cascade.

23. (Original) The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. (Original) The kit of claim 23, whereon the metered dose inhaler is a pressurized metered dose inhaler.

25. (Original) The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. (Original) The kit of claim 22, wherein said inhaler is a nebulizer.

27. (Original) The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

28. (Original) The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. (Original) The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. (Original) A pulmonary drug delivery kit comprising a) an anti C5 antibody composition comprising 1 mg/ml to 200 mg/ml; and b) a label indicating administration by nebulization.

REMARKS

Claims 15-30 are pending and claims 1-14 are withdrawn.

Applicant has amended claims 15 and 18 to improve its form and to more particularly point out the claimed invention. The amended claims are fully supported by the specification (e.g., Examples 2 and 3) and originally filed claims 15 and 18.

Accordingly, no new matter has been introduced. Applicant respectfully requests reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

DETAILED ACTION

Election/Restriction

1. Applicant acknowledges that claims 1-14 are withdrawn.

Claim Objections

2. Claims 18-21 are objected to because claim 18 is allegedly ambiguous in reciting "wherein the package is suitable for preventing or treating a pulmonary condition in a subject." Applicant has amended claim 18 to recite that the antibody is suitable, thus obviating the objection.

Claim Rejections Under 35 U.S.C. § 112

3. Claims 15-21 are rejected under 35 U.S.C. § 112 for allegedly not providing enablement for preventing asthma or other pulmonary conditions. Applicant disagrees.

Nevertheless, solely in an effort to expedite prosecution, Applicant has amended claims 15 and 18 to remove the recitation of "preventing," thus obviating the rejection.

Rejection Under 35 U.S.C. § 103

4. Claims 15, 16 and 18-20 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795 and US Patent No. 5,871,734. According to the Office Action, it would have been obvious to a person of ordinary skill in the art to use a nebulizer to administer an antibody that inhibits C5a to treat subjects with asthma based on the teachings of the cited references. Applicant respectfully traverses the rejection.

The Drouin reference is directed to characterizing C3aR and C5aR expression on bronchial epithelial and smooth muscles cells in view of their participation in the pathology of sepsis and asthma. (See Drouin, e.g., at Abstract and page 2026, column 1, paragraph 1). Applicant submits that Drouin does not disclose or suggest methods for using an anti-C5 antibody as claimed herein.

Drouin et al. does not disclose or even suggest methods for using an anti-C5 antibody as specified by the subject claims. Rather, Drouin et al. discloses a series of experiments characterizing the protein expression of C3aR and C5aR on bronchial epithelial and smooth muscle cells in murine models of sepsis and asthma. The data provided in this article clearly show that C3aR was upregulated in both the endotoxemia (LPS) model and the OVA-challenged mouse model but C5aR was upregulated ***only*** in the LPS model (see final paragraph on page 2028 continuing onto page 2029). It was known in the art and stated by the authors of Drouin et al. that LPS-induced inflammation in mouse is used as a model for endotoxemia, whereas OVA-induced inflammation in mouse lungs is used as a model for asthma. (See, e.g., Drouin et al. at page 2028, column 2 to page 2029, column 2).

Although Drouin states that "C5aR staining was detected on granulocytes and macrophages recruited to the lung as a result of the OVA challenge[,]" Applicants point out that the authors are silent regarding the significance of this observation. In fact, the absence of increased expression of C5aR on bronchial epithelial and smooth muscles cells in OVA-induced asthma – the primary focus of the reference (see above) – led the authors to conclude that:

> [a]lthough further study is required to determine the role of each receptor in these inflammatory models, ***these results suggest that both receptors contribute to lung function in the endotoxemia model, whereas C3aR may play a more significant role in lung inflammation in the asthma model.*** Recent observations that OVA-challenged C3aR-deficient guinea pigs (65) and mice (66) have reduced bronchial hyperactivity support the concept that C3aR may regulate bronchial smooth muscle function in this disease. (See Drouin at page 2031, column 1, paragraph 2; emphasis added).

In other words, their data suggests that activation of C3aR by C3a may be the more dominant mediator of lung inflammation in asthma.

One of skill in the art at the time of filing would recognize that in the complement cascade, the generation and activity of complement component C3a occurs before that of complement component C5a. This means that an inhibitor of C5 does not affect production of C3a. Accordingly, the skilled artisan reading Drouin would **not** have believed that inhibition of C5 or C5a would treat or prevent a C3a-mediated disease like asthma. Rather, the skilled artisan would have believed that inhibition of C3a or C3aR would be beneficial for treating asthma. In support of this assertion, Applicant provides Humbles et al. (Nature (2000) 406:998-1001; a copy of which is enclosed as Exhibit D), which is a reference published five months prior to Drouin. The reference demonstrates that mice deficient in C3aR alone were protected against methacholine-induced airway hyperresponsiveness in a mouse model of asthma. (See Humbles et al., e.g., at page 999, Figure 3). For at least these reasons, Applicant submits that the skilled artisan at the time of filing reading Drouin would not have believed inhibition of C5 would be beneficial for treating an asthma patient and thus would have had no reason to practice the claimed methods.

Furthermore, even if Drouin did disclose or suggest using anti-C5 antibodies to treat asthma, ***which it did not***, the prior art as a whole at the time of filing (as well as the post-filing references) actually taught away from benefits of using anti-C5 antibodies to treat asthma.

Applicant points out that a proper determination of *prima facie* obviousness requires that the Examiner consider ***all teachings in the analogous prior art*** and what the combined teachings would have suggested to the skilled artisan. The MPEP states that

> [w]here the teachings of two or more prior art references conflict, the examiner must weigh the power of each reference to suggest solutions to one of ordinary skill in the art, considering the degree to which one reference might accurately discredit another. See MPEP § 2143.01 (II) citing *In re Young*, 927 F.2d 588, 18 USPQ2d 1089 (Fed. Cir. 1991).

As evidence of the state of the art at the priority date of the application, Applicant provides Karp et al. (2000) *Nature Immunology* 1(3):221 (referred to as Karp; provided as Exhibit A), a reference published one year prior to Drouin. The authors of Karp found that lower expression of C5 protein is associated with more severe OVA-induced inflammation in mouse lung (see Figure 1). This discovery is in stark contrast to what a skilled artisan would have expected under the Examiner's interpretation of the Drouin publication.

In addition, Karp discloses that inhibition of C5aR results in a marked reduction in IL-12 production by monocytes *in vitro* (see Figure 3). IL-12 is a cytokine that is able to prevent or reverse experimental allergic asthma (See Abstract of Karp). This would lead one to believe that inhibition of C5 would exacerbate asthma.

Thus, Applicant submits that Karp **teaches away** from the notion that lower levels of C5 protein or activity would reduce the severity of OVA-induced inflammation in mouse lung. Accordingly, the skilled artisan reading Karp would have believed that C5 was protective against asthma and that inhibition of C5 or C5aR would not be beneficial for treating asthma.

Applicant also points out that in later publications, the authors of the Drouin reference also taught away from the use of C5 or C5aR inhibition for treating asthma. In 2006, Drs. Scott Drouin

Samsung Bioepis Exhibit No. 1002
Page 8440

and Rick Wetsel (the first and senior author on the Drouin et al. reference, respectively) co-authored a scientific publication demonstrating a protective role for C5 in allergic airway disease. (Drouin et al. (2006) *Am J Respir Crit Care Med* 173:852-857, a copy of which is enclosed as Exhibit B; referred to as "Drouin 2006"). Drouin 2006 disclosed that C5-deficient mice developed more severe asthma, including increased granulocyte infiltration of the lungs, than their C5-sufficient counterparts (see Figure 2). The authors also demonstrated that administration of an anti-C5 antibody (BB5.1) resulted in increased, ***not decreased***, airway hyperresponsiveness (AHR) in acetylcholine-challenged mice (see Figure 7).

Moreover, Drs. Drouin and Wetsel co-authored an Abstract disclosing a study on AHR and airway inflammation in C5aR knockout mice (Sinha et al. (2008) *Molecular Immunology* 45:4109-4110, Abstract No. O43, a copy of which is enclosed as Exhibit C). In Sinha et al., the authors disclose that C5aR knockout mice, as well as wild-type mice treated with a C5aR antagonist, exhibited exacerbated AHR as compared to wild-type or untreated mice, respectively. The authors state that "deficiency or antagonism of the C5aR in a mouse model of pulmonary allergy causes elevated AHR[.]" (See Sinha at page 4109, paragraph 2).

To summarize, the Drouin (2001) publication cited by the Examiner sets forth an observation that C5aR expressing cells (macrophages and granulocytes) are recruited into the lung in an animal model of asthma, but no relevance is made by the authors regarding that observation, the focus of Drouin (2001) being on expression of C3aR and C5aR on lung cells themselves. Additionally, both the prior art (as exemplified by the Humbles and Karp publications) and post-filing art (as exemplified by Drouin (2006) and Sinha et al. - notably both with authors from the Drouin (2001) publication) teach away from using a C5 inhibitor to treat asthma.

Therefore, contrary to the Examiner's assertions, the combination of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734, together with the teachings of the prior art as a whole at the time of filing, fails to render the claims obvious. That is, in view of the contradictory teachings of

the art, the skilled artisan would not have had reason to practice the claimed methods. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

5. Claims 17 and 21 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734 and US Patent No. 5,728,844. The Examiner states that Drouin does not teach an additional active agent, but that US Patent No. 5,728,844 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to add dexamethasone as an active agent. Applicant respectfully traverses this rejection.

The teachings of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 5,728,844. Neither reference teaches that C5aR is relevant to asthma. Thus, the references taken in combination do not teach or suggest all elements of claims 17 and 21. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

6. Claims 22 and 25-30 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734 and US Patent No. 6,524,836. The Examiner states that Drouin does not teach a kit or dry powder inhaler, but that US Patent No. 6,524,836 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to use the kit taught by US Patent No. 6,524,836 to package the active combination as taught by the combination of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734. Applicant respectfully traverses this rejection.

The teachings of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 6,524,836. Neither reference teaches that C5aR is relevant to asthma. Thus, the references taken in combination do not teach or suggest all elements of claims 22 and 25-30. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

7. Claims 23 and 24 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734, US Patent No. 6,524,836 and US Patent No. 6,905,688. The Examiner states that Drouin does not teach a metered dose inhaler, but that US Patent No. 6,905,688 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to use the metered inhaler in combination with the cited references. Applicant respectfully traverses this rejection.

The teachings of Drouin and US Patent Nos. 4,228,795, 5,871,734 and 6,524,836 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 6,905,688. Neither reference teaches that C5aR is relevant to asthma. Thus, the references taken in combination do not teach or suggest all elements of claims 23 and 24. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

CONCLUSION

In view of the foregoing amendments and remarks, Applicants submit that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. Applicant does not accede to any of the Examiner's positions not specifically addressed above. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Please charge any fees or credit any overpayments to our Deposit Account No. 18-1945 from which the undersigned is authorized to draw, under order no. ALXN-P02-102.

Dated: December 8, 2008

Respectfully submitted,

By Anita Varma

Anita Varma
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Attorneys/Agents For Applicant

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EM 111665494 US, on the date shown below in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: December 8, 2008 Signature: Pamela Harrison
(Pamela A. Harrison)

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang



Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

Examiner: F. P. Vandervegt

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (SIDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the references listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the references be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Supplemental Information Disclosure Statement is filed more than three months after the U.S. filing date, OR more than three months after the date of entry of the national stage of a PCT application, AND after the mailing date of the first Office Action on the merits, whichever occurs first, but before the mailing date of a Final Office Action or Notice of Allowance (37 CFR 1.97(c)).

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Samsung Bioepis Exhibit No. 1002
Page 8445

None of the documents in the attached form PTO/SB/08 are supplied because they were previously cited by or submitted to the Office in prior application number 10/655,861, filed September 5, 2003 and relied upon in this application for an earlier filing date under 35 U.S.C. 120.

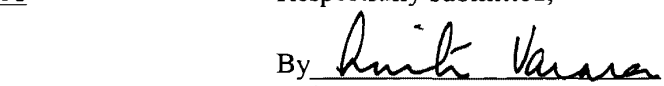
In accordance with 37 CFR 1.97(g), the filing of this Supplemental Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Supplemental Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Supplemental Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed references.

Please charge our Deposit Account No. 18-1945 in the amount of $180.00 covering the fee set forth in 37 CFR 1.17(p). The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: December 8, 2008

Respectfully submitted,

By 

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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F. P. Vandervegt
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
	CT1	Matis and Rollins, Complement-specific antibodies: Designing novel anti-inflammatories, Nature Medicine, Vol. 1 No. 8, 1995	

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EM 111665494 US, on the date shown below in an envelope addressed to:
MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: December 8, 2008 Signature: Pamela Harrison (Pamela A. Harrison)

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Samsung Bioepis Exhibit No. 1002
Page 8447

12-10-08

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AMENDMENT TRANSMITTAL LETTER

Docket No. ALXN-P02-102

Application No.	Filing Date	Examiner	Art Unit
11/127,438	May 11, 2005	F. P. Vandervegt	1644

Applicant(s): Yi Wang

Invention: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated and is transmitted as shown below.

CLAIMS AS AMENDED

	Claims Remaining After Amendment	Highest Number Previously Paid	Number Extra Claims Present	Rate	
Total Claims	31	- 31 =		x	
Independent Claims	5	- 5 =		x	
Multiple Dependent Claims (check if applicable)	☐				
Other fee (please specify):	Extension for response within first month; Submission of an Information Disclosure Statement				245.00
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT:					245.00

☐ Large Entity ☒ Small Entity

☐ No additional fee is required for this amendment.

☒ Please charge Deposit Account No. 18-1945 in the amount of $ 245.00. A duplicate copy of this sheet is enclosed.

☐ A check in the amount of $ _____ to cover the filing fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized to charge and credit Deposit Account No. 18-1945 as described below. A duplicate copy of this sheet is enclosed.

☒ Credit any overpayment.

☒ Charge any additional filing or application processing fees required under 37 CFR 1.16 and 1.17.

Anita Varma

Dated: December 8, 2008

Anita Varma, Esq.
Attorney/Agent Reg. No.: 43,221

ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110
(617) 951-7796

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Dated: December 8, 2008 Signature: Pamela Harrison (Pamela A. Harrison)

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Samsung Bioepis Exhibit No. 1002
Page 8448

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PTO/SB/17 (10-08)
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Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2009

[x] Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT	($) 245.00

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Examiner Name	F. P. Vandervegt
Art Unit	1644
Attorney Docket No.	ALXN-P02-102

METHOD OF PAYMENT (check all that apply)

[ ] Check [ ] Credit Card [ ] Money Order [ ] None [ ] Other (please identify): _____

[x] Deposit Account Deposit Account Number: 18-1945 Deposit Account Name: Ropes & Gray LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

[x] Charge fee(s) indicated below
[ ] Charge fee(s) indicated below, **except for the filing fee**
[x] Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17
[x] Credit any overpayments

FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
		Small Entity		Small Entity		Small Entity	
Application Type	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fee ($)	Fees Paid ($)
Utility	330	165	540	270	220	110	
Design	220	110	100	50	140	70	
Plant	220	110	330	165	170	85	
Reissue	330	165	540	270	650	325	
Provisional	220	110	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	Fee ($)	Small Entity Fee ($)
Each claim over 20 (including Reissues)	52	26
Each independent claim over 3 (including Reissues)	220	110
Multiple dependent claims	390	195

Total Claims	Extra Claims	Fee ($)	Fee Paid ($)
31	- 31 or HP = _____	x _____	= _____

HP = highest number of total claims paid for, if greater than 20.

Multiple Dependent Claims	
Fee ($)	Fee Paid ($)
_____	_____

Indep. Claims	Extra Claims	Fee ($)	Fee Paid ($)
5	- 5 or HP = _____	x _____	= _____

HP = highest number of independent claims paid for, if greater than 3.

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper (excluding electronically filed sequence or computer listings under 37 CFR 1.52(e)), the application size fee due is $270 ($135 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets	Extra Sheets	Number of each additional 50 or fraction thereof	Fee ($)	Fee Paid ($)
_____ - 100 =	_____ /50 =	_____ (round up to a whole number) x	_____	= _____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	
Other (e.g., late filing surcharge): 2251 Extension for response within first month	65.00
1806 Submission of an Information Disclosure Statement	180.00

SUBMITTED BY					
Signature	Anita Varma	Registration No. (Attorney/Agent)	43,221	Telephone	(617) 951-7796
Name (Print/Type)	Anita Varma, Esq.			Date	December 8, 2008

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EM 111665494 US, on the date shown below in an envelope addressed to:
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Dated: December 8, 2008 Signature: Pamela Harrison (Pamela A. Harrison)

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Page 8449

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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) ALXN-P02-102
Application Number 11/127,438	Filed May 11, 2005
For NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES	
Art Unit 1644	Examiner F. P. Vandervegt

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
[x]	One month (37 CFR 1.17(a)(1))	$130	$65	$ 65.00
[ ]	Two months (37 CFR 1.17(a)(2))	$490	$245	$
[ ]	Three months (37 CFR 1.17(a)(3))	$1110	$555	$
[ ]	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
[ ]	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

[x] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[ ] Payment by credit card. Form PTO-2038 is attached.

[x] The Director has already been authorized to charge fees in this application to a Deposit Account.

[x] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 18-1945 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71. Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

[x] attorney or agent of record. Registration Number 43,221

[ ] attorney or agent under 37 CFR 1.34. Registration number if acting under 37 CFR 1.34

Signature — December 8, 2008 (Date)

Anita Varma, Esq. (Typed or printed name) — (617) 951-7796 (Telephone Number)

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[x] Total of 1 forms are submitted.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EM 111665494 US, on the date shown below in an envelope addressed to: MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: December 8, 2008 Signature: Pamela Harrison (Pamela A. Harrison)

12/10/2008 WASFAN1 00000031 181945 11127438
01 FC:2252 245.00 DA

Samsung Bioepis Exhibit No. 1002
Page 8450

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
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PATENT APPLICATION FEE DETERMINATION RECORD
Substitute for Form PTO-875

Application or Docket Number	Filing Date	☐ To be Mailed
11/127,438	05/11/2005	

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	12/08/2008	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 31	Minus	** 31	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 5	Minus	***5	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/JOY DOBBS/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Samsung Bioepis Exhibit No. 1002
Page 8451

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/127,438	05/11/2005	Yi Wang	ALXN-P02-102	5259

28120 7590 03/19/2009
ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

EXAMINER	
VANDERVEGT, FRANCOIS P	
ART UNIT	PAPER NUMBER
1644	

MAIL DATE	DELIVERY MODE
03/19/2009	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/127,438	WANG, YI	
	Examiner	Art Unit	
	F. Pierre VanderVegt	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *08 December 2008*.

2a)☒ This action is **FINAL**. 2b)☐ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *1-30* is/are pending in the application.

4a) Of the above claim(s) *1-14* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *15-30* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☐ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *20081208*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

This application is a continuation-in-part of U.S. Application Serial Number 10/655,861, which claims the benefit of the filing date of provisional applications 60/408,571 and 60/469189.

Claims 1-30 are currently pending.

Election/Restrictions

1. **Claims 1-14 stand withdrawn from further consideration** pursuant to 37 CFR 1.142(b) as being drawn to a nonelected invention, there being no allowable generic or linking claim. Election was made **without** traverse in the reply filed on June 16, 2008.

Claims 15-30 read upon the elected invention and upon the elected species. Accordingly**, claims 15-30 are the subject of examination** in the present Office Action.

In view of Applicant's amendment filed December 8, 2008 only the following grounds of rejection are maintained.

Claim Rejections - 35 USC § 103

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

> (a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

2. Claims 15, 16, and 18-20 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CQ on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449) and U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892).

It was previously stated: "Drouin teaches that C5a receptors are increased on bronchial epithelial and smooth muscle cells in sepsis and in asthma (Abstract in particular). Drouin teaches that septic primates and rats that are treated with anti-C5a antibodies have reduced pulmonary edema and lung injury (page 2031, first column in particular).

Drouin does not teach a disperser for dispersing a medicament composition in an aerosol.

Samsung Bioepis Exhibit No. 1002
Page 8454

The '795 patent teaches a nebulizer which can be used to aerosolize medicants for nasal inhalation (Figure 4 and column 6, line 7 through column 8, line 54 in particular). The '795 patent further teaches that said nebulizer is suitable for use with viscous or sticky substances (column 8, lines 34-37 in particular). The '795 patent teaches that the nebulizer uses a jet of air to disperse the medicament (column 4, lines 35-39 in particular)[claims 18, 19]. The '795 patent also teaches the use of ultrasonic nebulizers in the art {column 1, lines 23-34 in particular)[claim 19].

As stated, the '795 patent further teaches that said nebulizer is suitable for use with viscous or sticky substances. The '734 patent specifically teaches that aqueous antibody solutions can be delivered to airways using a nebulizer (column 6, lines 36-41 and column 12, lines 37-52 in particular).

It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to use the nebulizer taught by the '795 patent to administer the anti-C5a antibodies taught by Drouin. One would have been motivated with a reasonable expectation of success to administer the antibodies directly to the respiratory mucosa, which is often the first line of encounter of an immune system with pathogenic organisms and by the teachings of the '795 patent that the nebulizer is usable with sticky substances, which a common property of proteinaceous solutions and the '734 patent teaches the effective delivery of antibodies to pulmonary tissue using a nebulizer.

Claim 20 further asserts a limitation of comprising instructions for the use of the composition. The printed matter on the label or package insert does not lend patentable weight as a limitation of the claimed invention because the recited intended use does not physically or chemically change the nature of the agent within the container and the artisan can still use the agent for other purposes. The intended use of a product, in this case an anti-C5 antibody, does not materially change the product as the product can still be used for other purposes, such as assaying C5 binding or immunopurification of C5, which are routinely practiced in the art. The recited "limitations" only further limit the intended use method printed on the label or package insert and do not change the physical and/or chemical properties of the components of the kit. In *In re* Haller (73 USPQ 403 (CCPA 1947)) the Court decided upon a case in which "it is not alleged by the Examiner or the board that the use of the involved compound as an insecticide is old or obvious and, in fact a claim covering broadly a process comprising the application of that compound to insects for the purpose of destroying them, has been allowed. In the instant case the appealed claim calls for a packaged product of an old compound labeled to indicate that the compound is intended for use as an insecticide." It is respectfully submitted that the facts of In re Haller are directly pertinent to the instant case. The method of treating an individual using compounds and compositions such as those contained in the instantly claimed kit has already been allowed in a parent application. Applicant relies therefore, as in In re Haller, upon printed matter reciting an intended use of the otherwise old compounds and compositions. The Court noted in *In re* Haller that "It is well settled that the application of particular printed matter to an old article cannot render the article patentable" and decided that, "the mere labeling of an old composition as an insecticide does not make it a new or different composition within the meaning of the patent statutes" and "Whether the statement of intended use appears merely in the claim or in a label on the product is immaterial so far as the question of patentability is concerned" in affirming the rejection. In *In re* Venezia (189 USPQ 149 (CCPA 1976)) the Court decided that the kit components were limited by the instructions listed in the claims because said kit components had to be adapted in a particular manner in order to meet the final assembly instructions that were part of the claim. The Court stated however that, "what may or may not happen in the future is not a part of the claimed invention" (emphasis by Court). The Court decided that the instructions of the claim were material to the claim because, "Rather than being a mere direction of activities to take place in the future, this language imparts a structural limitation to the sleeve" (emphasis added). The court reasoned this position by stating, "this language also defines present structures or attributes of the part of the 'kit' identified as the housing, which limits the structure of the housing to those configurations which allow for the completed connector assembly desired." It is respectfully submitted that, in the instant case, the

Samsung Bioepis Exhibit No. 1002
Page 8455

instructions do not modify the reagent comprised in the claimed kit, i.e., the reagent does not have to be altered for use commensurate with the instructions, rather the printed instructions merely set forth steps for what may be done with the kit components in the future. Accordingly, the printed instructions do not lend patentable weight to the claimed invention."

Applicant's arguments filed December 8, 2008 have been fully considered but they are not persuasive.

Applicant argues that Drouin does not "disclose or suggest methods for using an anti-C5 antibody as claimed herein."

Applicant's arguments are not convincing because the claimed invention is not drawn to any method, but is drawn to an aerosol composition and kits comprising same. Furthermore, Applicant argues that Drouin does not teach elevated C5 receptor in asthma and therefore does not teach the claimed method. However, Applicant is reminded that the claimed composition is for an effective amount to treat a pulmonary condition, not asthma. As stated previously, Drouin teaches that septic primates and rats that are treated with anti-C5a antibodies have reduced pulmonary edema and lung injury (page 2031, first column in particular). Accordingly, Drouin clearly teaches an effective amount for the treatment of a pulmonary condition. Applicant is further reminded that merely amending the claims to recite --asthma-- in lieu of "pulmonary condition" will not be sufficient to overcome the ground of rejection for at least two reasons. First, the composition would not be limited in its use for the treatment of ONLY asthma by such a recitation in the claims, as the artisan is free to use the composition for other purposes. Second, the claims are drawn to a composition "suitable for treating" a subject. There is no threshold provided in the claim regarding how effective the treatment with the composition would have to be. Accordingly, any amount of composition that an artisan would even expect to have a slight effect in a subject would satisfy the metes and bounds of the claims.

3. Claims 17 and 21 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CP on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449) and U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892) as applied to claims 15 and 18 above, and further in view of U.S. Patent No. 5,728,844 to Muller et al. (D on form PTO-892).

It was previously stated: "Drouin, the '795 patent and the '734 patent have been discussed supra. The combined references do not specifically teach an additional active agent.

Samsung Bioepis Exhibit No. 1002
Page 8456

The '844 patent teaches that it is common in the art to treat sepsis patients with dexamethasone in order to counter the effects of TNF.alpha. and that treatment with dexamethasone can be combined with the use of other medicaments (column 4, line 66 to column 5, line 20 in particular).

It would have been prima facie obvious to a person having ordinary skill in the art at the time the invention was made to add dexamethasone as an active agent in a treatment composition as taught by the combined teachings of Drouin, the '795 patent and the '734 patent. One would have been motivated to make the combination with a reasonable expectation of success by the teachings of the '844 patent that it is desirable to inhibit TNF.alpha. during the treatment of sepsis and that use of dexamethasone can be combined with the use of other medicaments."

Applicant has not provided substantive argument regarding the teachings of the '844 patent, relying instead upon the alleged deficiencies of the teachings of Drouin, which have been addressed supra. Accordingly, the instant ground of rejection stands without any further explanation.

4. Claims 22, and 25-30 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CP on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449) and U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892) as applied to claims 15 and 18 above, and further in view of U.S. Patent No. 6,524,836 to Sheppard (B on form PTO-892).

It was previously stated: "Drouin, the '795 and the '734 patent have been discussed supra.

The combined references do not specifically teach a kit or dry powder inhaler.

The '836 patent teaches kits comprising a nebulizer or dry powder inhaler for the delivery of medicaments to a subject, including medicaments comprising antibodies as an active agent (column 56, lines 48-62 in particular).

It would have been prima facie obvious to a person having ordinary skill in the art at the time the invention was made to package the active composition as taught by the combination of Drouin, the '795 and the '734 patent in a kit form as taught by the '836 patent.. One would have been motivated to assemble such a kit to make it easier for a practitioner or patient to store and use the components needed for treatment. One would have been further motivated to use a dry powder instead of a liquid suspension for similar reasons of storage and ease of use.

Claim 30 is included because, while the cited references do not specifically teach a concentration of anti-C5 antibodies to use in a pulmonary delivery composition, it would be routine well within the purview of one of ordinary skill in the art to determine such a concentration."

Applicant has not provided substantive argument regarding the teachings of the '836 patent, relying instead upon the alleged deficiencies of the teachings of Drouin, which have been addressed supra. Accordingly, the instant ground of rejection stands without any further explanation.

Samsung Bioepis Exhibit No. 1002
Page 8457

5. Claims 23 and 24 stand rejected under 35 U.S.C. 103(a) as being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032; CP on form PTO-1449 filed 9/25/2006) in view of U.S. Patent 4,228,795 to Babington (AC on form PTO-1449), U.S. Patent No. 5,871,734 to Lobb et al (A on form PTO-892) and U.S. Patent No. 6,524,836 to Sheppard (B on form PTO-892) as applied to claim 22 above, and further in view of U.S. Patent No. 6,905, 688 to Rosen et al. (C on form PTO-892).

It was previously stated: "Drouin, the '795 and the '734 patent have been discussed supra.
The combined references do not specifically teach a metered dose inhaler.
The '688 patent teaches the use of a metered dose inhaler as an alternative to the use of a nebulizer or dry powder inhaler for the aerosol delivery of a large protein medicament to a subject (column 17, lines 19-34 in particular). The '688 patent teaches that the composition can be supplied in the form of a kit (column 2, lines 51-61 in particular).
It would have been prima facie obvious to a person having ordinary skill in the art at the time the invention was made to use a metered dose inhaler as taught by the '688 patent in combination with the teachings of Drouin, the '795 and the '734 patent. One would have been motivated to use a metered dose inhaler as taught by the '688 patent with a reasonable expectation of success by the teachings of the '688 patent showing that a metered dose inhaler is an alternative to nebulizers and dry powder inhalers for the delivery of a medicament comprising a large protein active agent and perhaps by the need to have an exact measurement of the dose delivered to a subject, which may not be possible with a nebulizer or dry powder inhaler."

Applicant has not provided substantive argument regarding the teachings of the '688 patent, relying instead upon the alleged deficiencies of the teachings of Drouin, which have been addressed supra. Accordingly, the instant ground of rejection stands without any further explanation.

Conclusion

6. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action

7. No claim is allowed.

Samsung Bioepis Exhibit No. 1002
Page 8458

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to F. Pierre VanderVegt whose telephone number is (571)272-0852. The examiner can normally be reached on M-F 9:00-5:30.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Eileen O'Hara can be reached on (571) 272-0878. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

F. Pierre VanderVegt, Ph.D. /PV/
Patent Examiner
March 13, 2009

/Eileen B. O'Hara/
Supervisory Patent Examiner
Art Unit 1644

Samsung Bioepis Exhibit No. 1002
Page 8459

Index of Claims	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11127438	WANG, YI
	Examiner	Art Unit
	F. Pierre VanderVegt	1644

✓	Rejected	-	Cancelled	N	Non-Elected	A	Appeal
=	Allowed	÷	Restricted	I	Interference	O	Objected

☐ Claims renumbered in the same order as presented by applicant ☐ CPA ☐ T.D. ☐ R.1.47

CLAIM		DATE								
Final	Original	05/12/2008	08/28/2008	03/13/2009						
	1	÷	N	N						
	2	÷	N	N						
	3	÷	N	N						
	4	÷	N	N						
	5	÷	N	N						
	6	÷	N	N						
	7	÷	N	N						
	8	÷	N	N						
	9	÷	N	N						
	10	÷	N	N						
	11	÷	N	N						
	12	÷	N	N						
	13	÷	N	N						
	14	÷	N	N						
	15	÷	✓	✓						
	16	÷	✓	✓						
	17	÷	✓	✓						
	18	÷	✓	✓						
	19	÷	✓	✓						
	20	÷	✓	✓						
	21	÷	✓	✓						
	22	÷	✓	✓						
	23	÷	✓	✓						
	24	÷	✓	✓						
	25	÷	✓	✓						
	26	÷	✓	✓						
	27	÷	✓	✓						
	28	÷	✓	✓						
	29	÷	✓	✓						
	30	÷	✓	✓						

OIPE
DEC 08 2008
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PTO/SB/08b (11-08)
Approved for use through 12/31/2008. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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Substitute for form 1449/PTO

INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F. P. Vandervegt
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. [1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]
/PV/	CT1	Matis and Rollins, Complement-specific antibodies: Designing novel anti-inflammatories, Nature Medicine, Vol. 1 No. 8, 1995	

Examiner Signature	/F Pierre VanderVegt/	Date Considered	03/13/09

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1]Applicant's unique citation designation number (optional). [2]Applicant is to place a check mark here if English language Translation is attached.

I hereby certify that this paper (along with any paper referred to as being attached or enclosed) is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EM 111665494 US, on the date shown below in an envelope addressed to:
MS Amendment, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: December 8, 2008 Signature: Pamela Harrison (Pamela A. Harrison)

11463684_1.DOC

Samsung Bioepis Exhibit No. 1002
Page 8461

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11127438	WANG, YI
	Examiner	Art Unit
	F. Pierre VanderVegt	1644

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
PALM Inventor, 10/655,861, 60/408,571, 60/469,189 EAST, STN Medline Embase scisearch	8/26/08	PV
search strategy ATTACHED	8/28/08	PV

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

Electronic Acknowledgement Receipt	
EFS ID:	5539284
Application Number:	11127438
International Application Number:	
Confirmation Number:	5259
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Customer Number:	28120
Filer:	Ryan David Murphey/Andrea Silverman
Filer Authorized By:	Ryan David Murphey
Attorney Docket Number:	ALXN-P02-102
Receipt Date:	18-JUN-2009
Filing Date:	11-MAY-2005
Time Stamp:	11:04:06
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Document.pdf	2172866 b4f87d8c2ad71a476dc8b5b0fea5ee7aa02c11f0	yes	9

Samsung Bioepis Exhibit No. 1002
Page 8463

	Multipart Description/PDF files in .zip description		
	Document Description	**Start**	**End**
	Amendment After Final	1	1
	Claims	2	4
	Applicant Arguments/Remarks Made in an Amendment	5	9
Warnings:			
Information:			
	Total Files Size (in bytes):	2172866	

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Samsung Bioepis Exhibit No. 1002
Page 8464

PTO/SB/06 (07-06)
Approved for use through 1/31/2007. OMB 0651-0032
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Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 11/127,438	Filing Date 05/11/2005	☐ To be Mailed

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

AMENDMENT	06/18/2009	(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)	OR	OTHER THAN SMALL ENTITY RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	* 31	Minus	** 31	= 0	X $26 =	0	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 5	Minus	***5	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	0	OR	TOTAL ADD'L FEE	

AMENDMENT		(Column 1) CLAIMS REMAINING AFTER AMENDMENT		(Column 2) HIGHEST NUMBER PREVIOUSLY PAID FOR	(Column 3) PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)	OR	RATE ($)	ADDITIONAL FEE ($)
	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/DORIS m. BURNS/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

If you need assistance in completing the form, call 1-800-PTO-9199 and select option 2.

Samsung Bioepis Exhibit No. 1002
Page 8465

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438 | Confirmation No.: 5259

Filed: May 11, 2005 | Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES | Examiner: Vandervegt, Francois P.

RESPONSE TO FINAL OFFICE ACTION

MS AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Madam:

This Reply is filed in response to the Final Office Action mailed March 19, 2009 in the above-identified application. Reconsideration and further examination are requested.

Listing of the Claims begins on page 2 of this paper.

Remarks/Arguments begin on page 5 of this paper.

3931925_1.DOC

Samsung Bioepis Exhibit No. 1002
Page 8466

CLAIMS

This listing of claims will replace all prior versions and listings of claims in the application:

1. (Withdrawn) A method for preventing or treating a pulmonary condition in a subject comprising administering to the subject a therapeutically effective amount of an antibody that inhibits activation of the complement cascade and an additional active agent.

2. (Withdrawn) The method of claim 1, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD) interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

3. (Withdrawn) The method of claim 1, wherein the subject is a human.

4. (Withdrawn) The method of claim 1, wherein the antibody is specific to C5.

5. (Withdrawn) The method of claim 1, wherein the antibody is specific to C5 convertase.

6. (Withdrawn) The method of claim 1, wherein the antibody is specific to C5a or C5b-9.

7. (Withdrawn) The method of claim 1, wherein the antibody is eculizumab or pexelizumab.

8. (Withdrawn) The method of claim 1, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

9. (Withdrawn) The method of claim 7, wherein the mutated Fc portion does not activate complement.

Samsung Bioepis Exhibit No. 1002
Page 8467

10. (Withdrawn) The method of claim 1, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.

11. (Withdrawn) The method of claim 1, wherein the additional active agent is a corticosteroid.

12. (Withdrawn) The method of claim 1, wherein the corticosteroid is dexamethasone.

13. (Withdrawn) The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by the same delivery method or route.

14. (Withdrawn) The method of claim 1, wherein the antibody and the additional active agent may be administered to the subject by different delivery methods or routes.

15. (Previously Presented) An aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for treating a pulmonary condition in a subject.

16. (Original) The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. (Original) The aerosol composition of claim 15 further comprising an additional active agent.

18. (Previously Presented) A biopharmaceutical package comprising: a) an antibody that inhibits activation of the complement cascade; and b) a nebulizer, wherein the antibody is suitable for treating a pulmonary condition in a subject.

19. (Original) The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. (Original) The biopharmaceutical package of claim 19 further comprising instructions for use.

21. (Original) The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. (Previously Presented) A pulmonary drug delivery kit comprising: a) an inhaler; and b) an antibody formulated for inhalation delivery, wherein said antibody inhibits activation of the complement cascade.

23. (Original) The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. (Original) The kit of claim 23, whereon the metered dose inhaler is a pressurized metered dose inhaler.

25. (Original) The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. (Original) The kit of claim 22, wherein said inhaler is a nebulizer.

27. (Original) The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

28. (Original) The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. (Original) The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. (Original) A pulmonary drug delivery kit comprising a) an anti C5 antibody composition comprising 1 mg/ml to 200 mg/ml; and b) a label indicating administration by nebulization.

Samsung Bioepis Exhibit No. 1002
Page 8469

REMARKS

Claims 15-30 are pending and claims 1-14 are withdrawn. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

DETAILED ACTION

Election/Restriction

1. Applicant acknowledges that claims 1-14 are withdrawn.

Rejection Under 35 U.S.C. § 103

2. Claims 15, 16 and 18-20 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795 and US Patent No. 5,871,734. According to the Office Action, it would have been obvious to a person of ordinary skill in the art to use a nebulizer to administer an antibody that inhibits C5a to treat subjects with asthma based on the teachings of the cited references. Applicant respectfully traverses the rejection.

According to the Office Action, "Drouin teaches that septic primates and rats that are treated with anti-C5a antibodies have reduced pulmonary edema and lung injury[.]" (See Office Action at page 2.) However, Applicant respectfully points out that the subject claims are drawn to aerosol compositions containing antibodies that *inhibit activation of the complement cascade*. An anti-C5a antibody is not an antibody that inhibits activation of the complement cascade. A skilled artisan at the priority date of the application would have recognized that the production of C5a from complement component C5 and the production of C3a from complement component C3 are terminal events in the complement cascade. Accordingly, the artisan would have understood that inhibition of C5a using an anti-C5a antibody would not inhibit activation of the complement

cascade (e.g., the formation of C5a) as required by the claims. Drouin does not disclose or suggest an aerosol composition comprising an antibody that **inhibits activation** of the complement cascade, e.g., an antibody that prevents formation of C3a and/or C5a as claimed herein. Again, an antibody that directly inhibits C3a or C5a is not an antibody that **inhibits activation** of the complement cascade. Drouin merely discloses a series of experiments characterizing the protein expression of C3aR and C5aR on bronchial epithelial and smooth muscle cells in murine models of sepsis and asthma. (See Drouin, e.g., at Abstract and page 2026, column 1, paragraph 1).

It must be noted that administration of, e.g., anti-C5 monoclonal antibody, directly to the lungs has no systemic effect, i.e., it is active only in the lungs and not, e.g., in the serum. See, e.g., page 41, line 30, through page 42, line 2, of the application which states, "Aerosol administration of anti-C5 mAb, anti-C5 Fab, or anti-C5aR sIgG did not have any impact on serum C5b-9 mediated hemolytic activity." In order for one of ordinary skill in the art to have been motivated to treat a pulmonary condition with an aerosol composition comprising an antibody that **inhibits activation** of the complement cascade, the prior art would have had to teach that C3a and C5a are formed in the lungs. If C3a and C5a were already formed upon entry into the lungs, the antibody delivered by aerosol would be ineffective. Drouin does not teach that C3a and C5a are formed in the lungs. Drouin only states at the beginning of the second full paragraph on page 2031, "[i]nterestingly, bronchoalveolar lavage fluid from human patients with asthma or respiratory distress syndrome have been shown to contain C3a and C5a anaphylatoxins. These peptides **might potentially** originate from complement components produced by cells in the lung and then cleaved upon complement activation. Previous studies have demonstrated that resident alveolar macrophages and type II alveolar epithelial cells can synthesize the necessary components for complement system activation." (emphasis added; references deleted). One of ordinary skill in the art at the time of filing would not have taken this statement for a suggestion that C3a and C5a are formed in the lungs but merely a statement of the possibility.

Even if C3a and C5a were known to be formed in the lungs, that does not mean that additional C3a and C5a could not also enter the lungs from elsewhere, e.g. through the blood stream. If C3a and C5a formed and entered the lung from outside, antibodies that inhibit activation

of complement would still be ineffective in inhibiting such C3a and C5a if the antibodies are delivered directly to the lung as an aerosol. Therefore, one of ordinary skill in the art at the time of filing would not have been motivated to treat a pulmonary condition with an aerosol composition of an antibody that inhibits activation of the complement cascade without knowing that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. There is no indication in the cited references that either of these prerequisites was known.

Finally, an antibody that **inhibits activation** of the complement cascade as claimed encompasses inhibiting C5b formation. Drouin discusses C3aR and C5aR expression and further discusses anti-C5a antibodies, but this is irrelevant to the activity of C5b which can form in the absence of an inhibitor and which can form in the presence of an anti-C5a antibody. C5b forms part of the C5b-9 complex which is involved in causing symptoms of asthma. (See, e.g., page 14, lines 12-21 and page 16, lines 1-6 of the specification). Drouin does not teach or suggest inhibition of C5b. The claimed composition prevents formation of both C5a and C5b by blocking the complement cascade at or prior to cleavage of C5. Therefore, the claimed treatment will be improved as compared to the use of, e.g., an anti-C5a antibody alone.

In summary, Applicant respectfully submits that neither Drouin, US Patent No. 4,228,795 nor US Patent No. 5,871,734, alone or combination, render the claims obvious. Moreover, there would have been no reason for the skilled artisan to combine the references and arrive at the claimed compositions and the references fail to teach or suggest each and every element of the claimed invention. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

3. Claims 17 and 21 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734 and US Patent No. 5,728,844. The Examiner states that Drouin does not teach an additional active agent, but that US Patent No. 5,728,844 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to add dexamethasone as an active agent. Applicant respectfully traverses this rejection.

Samsung Bioepis Exhibit No. 1002
Page 8472

The teachings of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 5,728,844. Neither reference teaches that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. Furthermore, the references do not disclose inhibition of C5b formation. Thus, the references taken in combination do not teach or suggest all elements of claims 17 and 21. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

4. Claims 22 and 25-30 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734 and US Patent No. 6,524,836. The Examiner states that Drouin does not teach a kit or dry powder inhaler, but that US Patent No. 6,524,836 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to use the kit taught by US Patent No. 6,524,836 to package the active combination as taught by the combination of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734. Applicant respectfully traverses this rejection.

The teachings of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 6,524,836. Neither reference teaches that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. Furthermore, the references do not disclose inhibition of C5b formation. Thus, the references taken in combination do not teach or suggest all elements of claims 22 and 25-30. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

5. Claims 23 and 24 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734, US Patent No. 6,524,836 and US Patent No. 6,905,688. The Examiner states that Drouin does not teach a metered dose inhaler, but that US Patent No. 6,905,688 does. The

Examiner argues that it would have been obvious to a person of ordinary skill in the art to use the metered inhaler in combination with the cited references. Applicant respectfully traverses this rejection.

The teachings of Drouin and US Patent Nos. 4,228,795, 5,871,734 and 6,524,836 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 6,905,688. Neither reference teaches that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. Furthermore, the references do not disclose inhibition of C5b formation. Thus, the references taken in combination do not teach or suggest all elements of claims 23 and 24. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

CONCLUSION

In view of the foregoing remarks, Applicant submits that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. Applicant does not accede to any of the Examiner's positions not specifically addressed above. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Please charge any fees or credit any overpayments to our Deposit Account No. 18-1945 from which the undersigned is authorized to draw, under order no. ALXN-P02-102.

Dated: June 18, 2009

Respectfully submitted,

By _____
Ryan Murphey
Registration No.: 61,156
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

PTO/SB/30 (06-09)
Approved for use through 06/30/2009. OMB 0651-0031
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Request for Continued Examination (RCE) Transmittal

Address to:
Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F.P. Vandervegt
Attorney Docket Number	ALXN-P02-102

This is a Request for Continued Examination (RCE) under 37 CFR 1.114 of the above-identified application.

Request for Continued Examination (RCE) practice under 37 CFR 1.114 does not apply to any utility or plant application filed prior to June 8, 1995, or to any design application. See Instruction Sheet for RCEs (not to be submitted to the USPTO) on page 2.

1. Submission required under 37 CFR 1.114 Note: If the RCE is proper, any previously filed unentered amendments and amendments enclosed with the RCE will be entered in the order in which they were filed unless applicant instructs otherwise. If applicant does not wish to have any previously filed unentered amendment(s) entered, applicant must request non-entry of such amendment(s).

   a. [x] Previously submitted. If a final Office action is outstanding, any amendments filed after the final Office action may be considered as a submission even if this box is not checked.

      i. [ ] Consider the arguments in the Appeal Brief or Reply Brief previously filed on _____

      ii. [x] Other Response dated June 18, 2009

   b. [x] Enclosed

      i. [x] Amendment/Reply

      ii. [x] Affidavit(s)/Declaration(s)

      iii. [ ] Information Disclosure Statement (IDS)

      iv. [ ] Other _____

2. Miscellaneous

   a. [ ] Suspension of action on the above-identified application is requested under 37 CFR 1.103(c) for a period of _____ months. (Period of suspension shall not exceed 3 months; Fee under 37 CFR 1.17(i) required)

   b. [ ] Other _____

3. Fees The RCE fee under 37 CFR 1.17(e) is required by 37 CFR 1.114 when the RCE is filed.

   a. [x] The Director is hereby authorized to charge the following fees, any underpayment of fees, or credit any Overpayments, to Deposit Account No. 18-1945 .

      i. [x] RCE fee required under 37 CFR 1.17(e)

      ii. [x] Extension of time fee (37 CFR 1.136 and 1.17)

      iii. [ ] Other _____

   b. [ ] Check in the amount of $ _____ enclosed

   c. [ ] Payment by credit card (Form PTO-2038 enclosed)

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT REQUIRED

Signature	/Ryan Murphey/	Date	September 21, 2009
Name (Print/Type)	Ryan Murphey, Ph.D.	Registration No.	61,156

3994017_1.DOC

Samsung Bioepis Exhibit No. 1002
Page 8475

Electronic Patent Application Fee Transmittal

Application Number:	11127438
Filing Date:	11-May-2005
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Filer:	Ryan David Murphey/Andrea Silverman
Attorney Docket Number:	ALXN-P02-102

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Claims in excess of 20	1202	1	52	52
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Samsung Bioepis Exhibit No. 1002
Page 8476

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
Request for continued examination	1801	1	810	810
		Total in USD ($)		**862**

Samsung Bioepis Exhibit No. 1002
Page 8477

Electronic Acknowledgement Receipt	
EFS ID:	6107170
Application Number:	11127438
International Application Number:	
Confirmation Number:	5259
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Customer Number:	28120
Filer:	Ryan David Murphey/Andrea Silverman
Filer Authorized By:	Ryan David Murphey
Attorney Docket Number:	ALXN-P02-102
Receipt Date:	21-SEP-2009
Filing Date:	11-MAY-2005
Time Stamp:	12:08:38
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$862
RAM confirmation Number	7869
Deposit Account	181945
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.16 (National application filing, search, and examination fees) Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees)	

Samsung Bioepis Exhibit No. 1002
Page 8478

Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.20 (Post Issuance fees)

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		Response.pdf	129373 7c82987d6e46b821ef55918ed115631f018981e4	yes	10

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment Submitted/Entered with Filing of CPA/RCE	1	1
Claims	2	4
Applicant Arguments/Remarks Made in an Amendment	5	10

Warnings:

Information:

2	Rule 130, 131 or 132 Affidavits	Declaration.pdf	882672 9b6f12f03321e1ceb20d5cab0b713baac05d5406	no	2

Warnings:

Information:

3	Request for Continued Examination (RCE)	RCE.pdf	92573 85bd6deaa737a34ea8e99016e6b602969336252f	no	1

Warnings:

This is not a USPTO supplied RCE SB30 form.

Information:

4	Fee Worksheet (PTO-875)	fee-info.pdf	32038 30c49f4559cf80728401f62aa66f92da45f03709	no	2

Warnings:

Information:

Total Files Size (in bytes):	1136656

Samsung Bioepis Exhibit No. 1002
Page 8479

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New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Samsung Bioepis Exhibit No. 1002
Page 8480

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Yi Wang

Application No.: 11/127,438 | Confirmation No.: 5259

Filed: May 11, 2005 | Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES | Examiner: Vandervegt, Francois P.

AMENDMENT AND RESPONSE TO FINAL OFFICE ACTION ACCOMPANYING REQUEST FOR CONTINUED EXAMINATION

MS RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

In further response to the Final Office Action mailed March 19, 2009, applicants are filing a Request for Continued Examination under 37 C.F.R. § 1.114 of the above-identified application and are providing the following remarks which are supplementary to the applicant's June 18, 2009 Response, now entered with the request for continued examination. A request for a Three Month Extension of time is filed herewith. Thus, this response is timely filed. Reconsideration and further examination are requested.

Listing of the Claims begins on page 2 of this paper.

Remarks/Arguments begin on page 5 of this paper.

CLAIMS

This listing of claims will replace all prior versions and listings of claims in the application:

1-14. (Cancelled).

15. (Previously Presented) An aerosol composition comprising an antibody that inhibits activation of the complement cascade, wherein the composition is suitable for treating a pulmonary condition in a subject.

16. (Original) The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. (Original) The aerosol composition of claim 15 further comprising an additional active agent.

18. (Previously Presented) A biopharmaceutical package comprising: a) an antibody that inhibits activation of the complement cascade; and b) a nebulizer, wherein the antibody is suitable for treating a pulmonary condition in a subject.

19. (Original) The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. (Original) The biopharmaceutical package of claim 19 further comprising instructions for use.

21. (Original) The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. (Previously Presented) A pulmonary drug delivery kit comprising: a) an inhaler; and b) an antibody formulated for inhalation delivery, wherein said antibody inhibits activation of the complement cascade.

23. (Original) The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. (Currently Amended) The kit of claim 23, wherein ~~whereon~~ the metered dose inhaler is a pressurized metered dose inhaler.

25. (Original) The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. (Original) The kit of claim 22, wherein said inhaler is a nebulizer.

27. (Original) The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

28. (Original) The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. (Original) The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. (Original) A pulmonary drug delivery kit comprising a) an anti C5 antibody composition comprising 1 mg/ml to 200 mg/ml; and b) a label indicating administration by nebulization.

31. (New) A method for preventing or treating a pulmonary condition in a subject, the method comprising administering to the subject an effective amount of the aerosol composition of claim 15 to prevent or treat the pulmonary condition.

32. (New) The method of claim 31 wherein an additional active agent is administered.

33. (New) The method of claim 31, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema, bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

34. (New) The method of claim 31, wherein the subject is a human.

35. (New) The method of claim 31, wherein the antibody is specific to C5.

36. (New) The method of claim 31, wherein the antibody is specific to C5 convertase.

37. (New) The method of claim 31, wherein the antibody is specific to C5a or C5b-9.

38. (New) The method of claim 31, wherein the antibody is eculizumab or pexelizumab.

39. (New) The method of claim 31, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

40. (New) The method of claim 39, wherein the mutated Fc portion does not activate complement.

41. (New) The method of claim 31, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.

42. (New) The method of claim 32, wherein the additional active agent is a corticosteroid.

43. (New) The method of claim 42, wherein the corticosteroid is dexamethasone.

44. (New) The method of claim 32, wherein the antibody and the additional active agent are administered to the subject by the same delivery method or route.

45. (New) The method of claim 32, wherein the antibody and the additional active agent are administered to the subject by different delivery methods or routes.

REMARKS

Applicant cancels, without prejudice, claims 1-14. Applicant reserves the right to prosecute claims with identical or similar scope in one or more future continuation or divisional applications.

Claim 24 has been amended to replace "whereon" with "wherein." The amended claim is fully supported by the specification.

Applicant has added new claims 31-45. The new claims are fully supported by the specification and original claims 1-14. No new matter has been introduced.

Applicant's cancellation of and amendments to the claims are not in acquiescence to any of the Examiner's rejections. Applicant reserves the right to further prosecute the same or similar claims in the instant or in one or more subsequent patent applications claiming priority to the instant application.

After entry of amendments, claims 15-45 are pending. Applicant respectfully requests reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

DETAILED ACTION

Election/Restriction

1. Applicant acknowledges that claims 1-14 are withdrawn.

Rejection Under 35 U.S.C. § 103

2. Claims 15, 16 and 18-20 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795 and US Patent No. 5,871,734. According to the Office Action, it would have been obvious to a

person of ordinary skill in the art to use a nebulizer to administer an antibody that inhibits C5a to treat subjects with asthma based on the teachings of the cited references. Applicant respectfully traverses the rejection.

According to the Office Action, "Drouin teaches that septic primates and rats that are treated with anti-C5a antibodies have reduced pulmonary edema and lung injury[.]" (See Office Action at page 2.) However, Applicant respectfully points out that the subject claims are drawn to aerosol compositions containing antibodies that *inhibit activation of the complement cascade*. An anti-C5a antibody is not an antibody that inhibits activation of the complement cascade. A skilled artisan at the priority date of the application would have recognized that the production of C5a from complement component C5 and the production of C3a from complement component C3 are terminal events in the complement cascade. Accordingly, the artisan would have understood that inhibition of C5a using an anti-C5a antibody would not inhibit activation of the complement cascade (e.g., the formation of C5a) as required by the claims. Drouin does not disclose or suggest an aerosol composition comprising an antibody that **inhibits activation** of the complement cascade, e.g., an antibody that prevents formation of C3a and/or C5a as claimed herein. Again, an antibody that directly inhibits C3a or C5a is not an antibody that **inhibits activation** of the complement cascade. Drouin merely discloses a series of experiments characterizing the protein expression of C3aR and C5aR on bronchial epithelial and smooth muscle cells in murine models of sepsis and asthma. (See Drouin, e.g., at Abstract and page 2026, column 1, paragraph 1).

It must be noted that administration of, e.g., anti-C5 monoclonal antibody, directly to the lungs has no systemic effect, i.e., it is active only in the lungs and not, e.g., in the serum. See, e.g., page 41, line 30, through page 42, line 2, of the application which states, "Aerosol administration of anti-C5 mAb, anti-C5 Fab, or anti-C5aR sIgG did not have any impact on serum C5b-9 mediated hemolytic activity." In order for one of ordinary skill in the art to have been motivated to treat a pulmonary condition with an aerosol composition comprising an antibody that **inhibits activation** of the complement cascade, the prior art would have had to teach that C3a and C5a are formed in the lungs. If C3a and C5a were already formed upon entry into the lungs, the antibody delivered by aerosol would be ineffective. Drouin does not teach that C3a and C5a are formed in the lungs.

Drouin only states at the beginning of the second full paragraph on page 2031, "[i]nterestingly, bronchoalveolar lavage fluid from human patients with asthma or respiratory distress syndrome have been shown to contain C3a and C5a anaphylatoxins. These peptides **might potentially** originate from complement components produced by cells in the lung and then cleaved upon complement activation. Previous studies have demonstrated that resident alveolar macrophages and type II alveolar epithelial cells can synthesize the necessary components for complement system activation." (emphasis added; references deleted). One of ordinary skill in the art at the time of filing would not have taken this statement for a suggestion that C3a and C5a are formed in the lungs but merely a statement of the possibility.

Even if C3a and C5a were known to be formed in the lungs, that does not mean that additional C3a and C5a could not also enter the lungs from elsewhere, e.g. through the blood stream. If C3a and C5a formed and entered the lung from outside, antibodies that inhibit activation of complement would still be ineffective in inhibiting such C3a and C5a if the antibodies are delivered directly to the lung as an aerosol. Importantly, it must be noted that Drouin teaches that, "Staining for both receptors on pulmonary blood vessels from OVA-challenged mice was difficult to assess due to the massive influx of granulocytes and macrophages" (see middle of right-hand column on page 2029). Thus, in the OVA-induced asthma model it is clear that there is a massive influx into the lungs of cells from outside of the lungs. If the cells entering the lungs have already produced C3a and C5a, then administration of a composition to inhibit activation of the complement cascade will have no effect on that C3a and C5a and should be inadequate to treat the asthma. Therefore, one of ordinary skill in the art at the time of filing would not have been motivated to treat a pulmonary condition with an aerosol composition of an antibody that inhibits activation of the complement cascade without knowing that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. There is no indication in the cited references that either of these prerequisites was known.

Finally, an antibody that **inhibits activation** of the complement cascade as claimed encompasses inhibiting C5b formation. Drouin discusses C3aR and C5aR expression and further discusses anti-C5a antibodies, but this is irrelevant to the activity of C5b which can form in the

absence of an inhibitor and which can form in the presence of an anti-C5a antibody. C5b forms part of the C5b-9 complex which is involved in causing symptoms of asthma. (See, e.g., page 14, lines 12-21 and page 16, lines 1-6 of the specification). Drouin does not teach or suggest inhibition of C5b. The claimed composition prevents formation of both C5a and C5b by blocking the complement cascade at or prior to cleavage of C5. Therefore, the claimed treatment will be improved as compared to the use of, e.g., an anti-C5a antibody alone.

In summary, Applicant respectfully submits that neither Drouin, US Patent No. 4,228,795 nor US Patent No. 5,871,734, alone or combination, render the claims obvious. Moreover, there would have been no reason for the skilled artisan to combine the references and arrive at the claimed compositions and the references fail to teach or suggest each and every element of the claimed invention. Accordingly, reconsideration and withdrawal of this rejection are respectfully requested.

3. Claims 17 and 21 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734 and US Patent No. 5,728,844. The Examiner states that Drouin does not teach an additional active agent, but that US Patent No. 5,728,844 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to add dexamethasone as an active agent. Applicant respectfully traverses this rejection.

The teachings of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 5,728,844. Neither reference teaches that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. Furthermore, the references do not disclose inhibition of C5b formation. Thus, the references taken in combination do not teach or suggest all elements of claims 17 and 21. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

4. Claims 22 and 25-30 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734 and US Patent No. 6,524,836. The Examiner states that Drouin does not teach a kit or dry powder inhaler, but that US Patent No. 6,524,836 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to use the kit taught by US Patent No. 6,524,836 to package the active combination as taught by the combination of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734. Applicant respectfully traverses this rejection.

The teachings of Drouin, US Patent No. 4,228,795 and US Patent No. 5,871,734 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 6,524,836. Neither reference teaches that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. Furthermore, the references do not disclose inhibition of C5b formation. Thus, the references taken in combination do not teach or suggest all elements of claims 22 and 25-30. Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

5. Claims 23 and 24 are rejected under 35 U.S.C. § 103 (a) as allegedly being unpatentable over Drouin (J. Immunol. [2001] 166:2025-2032) in view of US Patent No. 4,228,795, US Patent No. 5,871,734, US Patent No. 6,524,836 and US Patent No. 6,905,688. The Examiner states that Drouin does not teach a metered dose inhaler, but that US Patent No. 6,905,688 does. The Examiner argues that it would have been obvious to a person of ordinary skill in the art to use the metered inhaler in combination with the cited references. Applicant respectfully traverses this rejection.

The teachings of Drouin and US Patent Nos. 4,228,795, 5,871,734 and 6,524,836 are discussed *supra*. The deficiencies of Drouin are not provided by US Patent No. 6,905,688. Neither reference teaches that C3a and C5a form in the lung and that no or only minimal C3a and C5a enter the lung from outside. Furthermore, the references do not disclose inhibition of C5b formation. Thus, the references taken in combination do not teach or suggest all elements of claims 23 and 24.

Accordingly, the claims are non-obvious over the cited prior art, and reconsideration and withdrawal of this rejection are respectfully requested.

Additionally, Applicant submits herewith a Declaration from Dr. Rick Wetsel, which was prepared to be submitted in the parent application, U.S. Patent Application No. 10/655,861. Dr. Wetsel is the corresponding author of the Drouin et al. reference cited in this Office Action as well. The Declaration is provided to clear up a possible misreading of the Drouin et al. publication by setting forth Dr. Wetsel's interpretation of certain language within the publication. Specifically, Dr. Wetsel indicates that only C3aR was shown to be upregulated on bronchial smooth muscle in the OVA-induced asthma model and not C5aR.

CONCLUSION

In view of the foregoing remarks, Applicant submits that the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. Applicant does not accede to any of the Examiner's positions not specifically addressed above. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Please charge any fees or credit any overpayments to our Deposit Account No. 18-1945 from which the undersigned is authorized to draw, under order no. ALXN-P02-102.

Dated: September 21, 2009

Respectfully submitted,

By /Ryan Murphey/
Ryan Murphey
Registration No.: 61,156
ROPES & GRAY LLP
One International Place
Boston, Massachusetts 02110-2624
(617) 951-7000
(617) 951-7050 (Fax)
Attorneys/Agents For Applicant

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Yi Wang

Serial No. 10/655,861 — Confirmation No.: 7250

Filed: 5 September 2003 — Examiner: F. P. VanderVegt

FOR: METHOD OF TREATMENT OF ASTHMA USING ANTIBODIES TO COMPLEMENT COMPONENT C5 — Group Art Unit: 1644

DECLARATION UNDER 37 CFR § 1.132

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Dr. Rick A. Wetsel, declare as follows:

1. I am the senior author of the publication "Expression of the Complement Anaphylatoxin C3a and C5a Receptors on Bronchial Epithelial and Smooth Muscle Cells in Models of Sepsis and Asthma" published by Drouin et al. in the *Journal of Immunology* 166:2025-2032 (2001).

2. I received a B.S. in Chemistry in 1976 from the University of Texas at Austin, a Ph.D. in Biochemistry in 1982 from the University of Texas Health Science Center at San Antonio, and I performed postdoctoral training in Immunology from 1983-1986 at the Scripps Research Institute, La Jolla, California. From 1986-1996 I was an Assistant Professor of Pediatrics and Assistant Professor of Molecular Microbiology, Washington University School of Medicine, Department of Pediatrics, St. Louis, Missouri. In 1992 I was a Visiting Investigator, Basel Institute for Immunology, Basel, Switzerland. From 1996-2001 I was an Associate Professor, Research Center for Immunology and Autoimmune Diseases, Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas-Houston, Houston, Texas; Director, Laboratory for Developmental Biology. From 2001-Present I have been Professor, Research Center for Immunology and Autoimmune Diseases, Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas-Houston; Director, Laboratory for Developmental Biology.

3. Alexion Pharmaceuticals, Inc., the Assignee of U.S. Patent Application Serial No. 10/655,861 entitled "Method of Treatment of Asthma Using Antibodies to Complement Component C5" has asked me to clarify the intended meaning of some statements in the Drouin et al. publication cited above for which I am the senior author. Specifically, the statements in the

Samsung Bioepis Exhibit No. 1002
Page 8491

first paragraph of the Discussion section on page 2029 may have been written less precisely than we intended, and this has apparently led to a conflict in the way that Alexion Pharmaceuticals interprets the statements as compared to the way the U.S. Patent and Trademark Office has interpreted the statements. Our intended meaning of the statements at issue is as follows.

4. The results disclosed in the Drouin et al. publication cited above show that C5aR is upregulated in the lung after LPS treatment; however, in the OVA model of allergic lung disease the C5aR was **not** upregulated. Instead, in the OVA model only the C3aR showed increased expression. Furthermore, C3aR is upregulated in the OVA model only on the smooth muscle cells.

5. The first paragraph of the Discussion on page 2029 of the cited Drouin et al. publication states, "This study documents for the first time the expression of C3aR by lung cells and confirms previous reports that cells endogenous to mouse and human lungs express C5aR. Moreover, we have established that both receptors are up-regulated in two distinct models of lung inflammation: endotoxemia and OVA-induced asthma." This opening sentence of the discussion was meant to be only general, with the more detailed differences discussed on pages 2030 and 2031. Since this opening sentence has caused some confusion, I wish we had written it in a more precise manner. In any event, on page 2031 we state the details: "In contrast to mice treated with LPS, C3aR and C5aR expression did not change on bronchial and alveolar epithelial cells from OVA-challenged lungs. However, bronchial smooth muscle expression of C3aR was increased in OVA-challenged mice relative to the saline controls."

6. The disagreement between Alexion Pharmaceuticals and the U.S. Patent and Trademark Office appears to be that concerning the language "Moreover, we have established that both receptors are up-regulated in two distinct models of lung inflammation: endotoxemia and OVA-induced asthma". One party's interpretation is that 1) C3aR and C5aR are both upregulated in both endotoxemia and OVA-induced asthma; whereas the second party interprets the language as 2) C3aR and C5aR are both upregulated in endotoxemia whereas only C3aR is upregulated in the OVA-induced asthma model. The second interpretation is correct. Only C3aR is upregulated in the OVA-induced asthma model and to be precise it was only the C3aR on bronchial smooth muscle that was increased in the OVA model. C5aR was not shown to be upregulated in the OVA-induced asthma model in the data presented in the above-cited Drouin et al. publication.

I further state that the above statements were made with the knowledge that willful false statements and the like are punishable by fine and/or imprisonment, or both, under Section 1001, Title 18 of the United States Code, and that any such willful false statement may jeopardize the validity of this application or any patent resulting therefrom.

Dated: May 21, 2009

Rick A. Wetsel

Rick A. Wetsel, Ph.D.

Samsung Bioepis Exhibit No. 1002
Page 8492

PTO/SB/22 (06-09)
Approved for use through 06/30/2009. OMB 0651-0031
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) ALXN-P02-102
Application Number 11/127,438	Filed May 11, 2005
For NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES	
Art Unit 1644	Examiner F. P. Vandervegt

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
☐	One month (37 CFR 1.17(a)(1))	$130	$65	$
☐	Two months (37 CFR 1.17(a)(2))	$490	$245	$
x	Three months (37 CFR 1.17(a)(3))	$1110	$555	$ 1,110.00
☐	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
☐	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

☐ Applicant claims small entity status. See 37 CFR 1.27.

☐ A check in the amount of the fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

x The Director has already been authorized to charge fees in this application to a Deposit Account.

x The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 18-1945 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

☐ applicant/inventor.

☐ assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

x attorney or agent of record. Registration Number 61,156

☐ attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Ryan Murphey/	September 21, 2009
Signature	Date
Ryan Murphey, Ph.D.	(212) 596-9000
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

x Total of 1 forms are submitted.

Electronic Patent Application Fee Transmittal

Application Number:	11127438
Filing Date:	11-May-2005
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Filer:	Ryan David Murphey/Andrea Silverman
Attorney Docket Number:	ALXN-P02-102

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				
Extension - 3 months with $0 paid	1253	1	1110	1110

Samsung Bioepis Exhibit No. 1002
Page 8494

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Miscellaneous:				
		Total in USD ($)		**1110**

Samsung Bioepis Exhibit No. 1002
Page 8495

Electronic Acknowledgement Receipt	
EFS ID:	6108123
Application Number:	11127438
International Application Number:	
Confirmation Number:	5259
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Customer Number:	28120
Filer:	Ryan David Murphey/Andrea Silverman
Filer Authorized By:	Ryan David Murphey
Attorney Docket Number:	ALXN-P02-102
Receipt Date:	21-SEP-2009
Filing Date:	11-MAY-2005
Time Stamp:	12:26:08
Application Type:	Utility under 35 USC 111(a)

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Samsung Bioepis Exhibit No. 1002
Page 8496

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Samsung Bioepis Exhibit No. 1002
Page 8497

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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 11/127,438	Filing Date 05/11/2005	☐ To be Mailed

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	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
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TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
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	Independent (37 CFR 1.16(h))	* 4	Minus	***5	= 0	X $110 =	0	OR	X $ =	
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		(Column 1)		(Column 2)	(Column 3)					
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Samsung Bioepis Exhibit No. 1002
Page 8498

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(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
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(54) Title: INCLUSION COMPLEXES OF BETA-2-ANDRENERGICS FOR ORAL MUCOSAL DELIVERY

(57) Abstract

A pharmaceutical composition for oral mucosal delivery comprises an active ingredient, an inclusion complex of (a) a selective β_2-adrenergic agonist or a pharmaceutically acceptable salt thereof such as salbutamol, and (b) an unsubstituted or substituted beta- or gamma-cyclodextrin, and a pharmaceutically acceptable carrier for oral mucosal delivery. The pharmaceutical composition is of use in the treatment of reversible obstructive airways diseases such as asthma.

Samsung Bioepis Exhibit No. 1002
Page 8500

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Samsung Bioepis Exhibit No. 1002
Page 8501

INCLUSION COMPLEXES OF BETA-2-ANDRENERGICS FOR ORAL MUCOSAL DELIVERY

BACKGROUND OF THE INVENTION

This invention relates to a pharmaceutical composition for oral mucosal delivery comprising as an active ingredient an inclusion complex of a selective $ß_2$-adrenergic agonist or a salt thereof, and a cyclodextrin.

Salbutamol(4-hydroxy-3-hydroxymethyl-α-[(*tert*-butylamino)methyl]-benzyl alcohol) and other selective $ß_2$-adrenergic agonists, such as metaproterenol, terbutaline, pirbuterol, salmeterol, fenbuterol, orciprenaline, formoterol, hexaprenaline, reproterol, rimiterol, fenoterol, procaterol, mabuterol, bambuterol and bitolterol, are useful for the treatment of reversible obstructive airways diseases such as asthma and the reversible component of chronic obstructive pulmonary diseases.

Asthma is a chronic disease in which the patient suffers episodes of reversible airways obstruction. It is a common disorder occurring in about 4-5% of the population. Fatalities can occur and mortality rates are reported to be increasing.

The underlying cause of asthma is poorly understood, but resistance to airflow is increased by a number of factors:

Samsung Bioepis Exhibit No. 1002
Page 8502

1 Constriction of airway smooth muscle

2 The presence of excessive secretions within the airway lumen

3 Thickening of the airway epithelium due to oedema, infiltration of the mucosa by eosinophils and other inflammatory cells, hypertrophy and hypertrophy and hyperplasia.

As asthma is a chronic disease, management involves prophylatic measures to reduce airway resistance and maintain airflow as well as specific regimens for the treatment of acute attacks.

The standard drugs used in the management of asthma are $ß_2$-adrenergic agonists. $ß_2$-adrenergic agonists relax the bronchial smooth muscle to produce bronchodilatation by stimulating $ß_2$-adrenergic receptors. Short acting selective $ß^2$-agonists such as salbutamol or terbutaline are the initial drugs of choice; if inhaled, they produce a bronchodilating effect within 15 minutes.

Initially, therapy is usually administered by inhalation to deliver the drugs to the desired site of action. The doses are approximately 5% of the dose required with oral administration. In severe asthma, the oral route may also be necessary. Difficulties associated with the use of inhalers may be overcome by the use of spacing devices to act as reservoirs for the drug to make it easier for the patient (especially a child) to inhale each dose.

Chronic obstructive pulmonary disease (COPD) covers a range of disorders of progressive airflow limitation including chronic bronchitis and emphysema. Unlike asthma, the obstruction of airflow is relatively constant and essentially irreversible. COPD is a common disorder, frequently associated with cigarette smoking, infections, environmental pollution and

Samsung Bioepis Exhibit No. 1002
Page 8503

occupational dust exposure. Drug treatment includes symptomatic and palliative therapy using bronchodilating agents. First-line drug therapy for the treatment of COPD consists of bronchodilating agents to alleviate bronchospasm and any reversible component of airway obstruction. The drug of first choice is a $ß_2$-selective agonist such as salbutamol or terbutaline given by inhalation.

As stated above, salbutamol and related $ß_2$-adrenergic agonists are administered either by inhalation or by the orogastric route. When administered by inhalation, salbutamol produces significant bronchodilation within 15 minutes and effects are demonstrable for 3 to 4 hours. With aerosol therapy only about 10% of the inhaled dose actually enters the lungs: much of the remainder is swallowed. Successful aerosol therapy requires that the patient masters the technique of drug administration. Many patients, particularly children and the elderly, do not use optimal techniques. Additionally, many allergic asthmatics suffer from mucous congestion of the respiratory tract which further reduces the absorption of inhaled bronchodilators.

Salbutamol is rapidly absorbed from the gastro-intestinal tract. It is subject to first-pass metabolism in the liver and possibly the gut wall. The main metabolite is an inactive sulphate conjugate. It is rapidly excreted in the urine as metabolites and unchanged drug; there is some excretion in the faeces. It has been suggested that the majority of an inhaled dose is swallowed and absorbed from the gut (see Goodman & Gilman's The Pharmacological Basis of Therapeutics, Eighth Edition, Volume 1, Chap. 10 p204). The plasma half-life of salbutamol has been estimated to range from about 2 to as much as 7 hours.

Samsung Bioepis Exhibit No. 1002
Page 8504

Administration of salbutamol by inhalation results in the rapid onset (within 5 - 15 minutes) of bronchodilation which lasts for about 4 hours. Following conventional orogastric administration, the onset of action is within 30 minutes, with a peak effect between 2 to 3 hours after the dose, and a duration of action of up to 6 hours (see MARTINDALE, The Extra Pharmacopoeia, 31ST Edition, p 1591).

Administration of drugs by direct absorption through the oral mucosa has several advantages, particularly rapid systemic uptake with fast onset of therapeutic action and avoidance of presystemic metabolism. Both of these advantages are especially of relevance to the use of β_2-adrenergic agonists in the treatment of reversible obstructive airways disease where rapid onset of action is desirable and where these compounds generally suffer extensive presystemic metabolism following orogastric administration. However, the low permeability of the membranes that line the oral cavity results in a low flux of the drug across the membrane. There is therefore a need to enhance the drug penetration to improve biovailability following oral mucosal drug delivery. There are several methods known in the art to deliver drugs to the oral mucosae. These include buccal and sublingual tablets or lozenges, adhesive patches, gels, solutions or sprays.

The absorption of drugs from mucosal membranes may be enhanced by (i) increasing drug solubility, (ii) pH modification to favour the unionized form of the drug, (iii) addition of mucoadhesive agents to improve contact between the delivery system and the membrane and (iv) incorporation of so-called penetration enhancers.

There are a number of penetration enhancers known to influence the permeability of drugs across epithelial membranes [for a recent review see

Samsung Bioepis Exhibit No. 1002
Page 8505

Walker, R.B. and Smith, E.W., Advanced Drug Delivery Reviews 1996, 18, 295-301].

Cyclodextrins and their derivatives have found extensive application as solubilizers and stabilizers due to their ability to form inclusion complexes with a wide variety of compounds [J. Szejtli, *Cyclodextrin Technology,* Kluwer Academic Press) and ([J. Szejtli & K-H Fromming, *Cyclodextrins in Pharmacy,* Kluwer Academic Press)]. Cyclodextrins have been used to enhance intestinal absorption of drugs primarily through increasing the solubility. Recently, cyclodextrins have been shown to have positive and negative effects on transdermal penetration of drugs [see Loftson, T. *et al,* International Journal of Pharmaceutics 1995, 115, 255-258), (Vollmer, U. *et al,* International Journal of Pharmaceutics 1993, 99, 51-58), (Legendre, J.Y. *et al,* European Journal of Pharmaceutical Sciences 1995, 3, 311-322) and (Vollmer, U. *et al,* Journal of Pharmacy and Pharmacology 1994, 46, 19-22)].

Cyclodextrins are water soluble cone-shaped cyclic oligosaccharides containing 6,7 or 8 glucopyranose units. The interior or "cavity" of the cone is hydrophobic whilst the exterior is hydrophillic. The size of the cavity increases with increasing number of glucose units. Several cyclodextrin derivatives such as alkyl, hydroxyalkyl and sulfoalkyl ethers have been prepared with improved solubility [see (J. Szejtli & K-H Fromming, *Cyclodextrins in Pharmacy,* Kluwer Academic Press) and (Stella, V.J. *et al,* Pharmaceutical Research 1995, 12 (9) S205)]. Suitably sized hydrophobic "guest" molecules may enter the "host" cavity to form a classical host-guest "inclusion compound" or "inclusion complex" with either the entire guest molecule included or only a portion thereof. The driving mechanism for cyclodextrin inclusion complexation is the affinity of the hydrophobic guest

Samsung Bioepis Exhibit No. 1002
Page 8506

molecule for the cavity of the cyclodextrin host molecule with displacement of cavity water molecules to form a thermodynamically more stable state. The term "complex stability" or stability of a given inclusion complex refers to the association/dissociation equilibrium of host and guest in solution. Complex stability depends on the number of intermolecular bonding interactions between the host and guest. Van der Waals forces and hydrophobic interactions are the main stabilising interactions in inclusion complexes (Bergeron, R.J. *et al,* Journal of the American Chemical Society 1977, 99, 5146). Depending on the nature and position of hydrogen bonding functionalities on a given guest, there may be hydrogen bonding between the guest and hydroxyl groups of the cyclodextrin or other hydrogen bonding groups in the case of cyclodextrin derivatives. Ionic interactions between the host and guest are also possible in the case of ionic cyclodextrins such as sulphobutyl ethers (Stella, V.J. *et al,* Pharmaceutical Research 1995. 12 (9) S205).

Cyclodextrin inclusion complexes may be prepared on the basis of liquid state, solid state or semi-solid state reactions between the components (J. Szejtli, *Cyclodextrin Technology,* Kluwer Academic Press). The first is accomplished by dissolving the cyclodextrin and guest in a suitable solvent or mixture of solvents and subsequently isolating the solid state complex by crystallisation, evaporation, spray drying or freeze drying. In the solid state method, the two components may be screened to uniform particle size and thoroughly mixed whereafter they are ground in a high energy mill with optional heating, screened and homogenized. In the semi-solid state reaction the two components are kneaded in the presence of small amounts of a suitable solvent, and the complex so-formed, is dried, screened and homogenized. The liquid state reaction generally provides optimum conditions for completeness.

Samsung Bioepis Exhibit No. 1002
Page 8507

Inclusion complexes of various cyclodextrins and salbutamol in both the liquid and the solid state have been reported in the literature [see (H.M. Cabal Marques, J. Hadgraft and I.W. Kellaway, *International Journal of Pharmaceutics*, 63 (1990) 259-266)]. Cabral Marques *et al* studied the interaction of salbutamol with beta-, alpha- and gamma-cyclodextrins. Using the methods of Higuchi and Connors (*Adv. Anal. Chem.*, 4(1965) 117-212) they determined by solution studies that the solubility of the salbutamol complexes increased in the order: Heptakis(2,6-di-*O*-methyl)-beta-cyclodextrin (DIMEB), > beta-cyclodextrin, >> gamma-cyclodextrin, >> alpha-cyclodextrin. This increase in solubility was attributed to the accessibility of the ring of the salbutamol molecule to interaction with the cyclodextrins. Overall, the cavity size of beta-cyclodextrins and its derivatives appear to be optimal for entrapment of the salbutamol molecule, and consequently provides the greatest solubilization effect. The authors also found that changes of temperature in the range 20-37°C had only a minimal effect on the solubility of salbutamol in both the absence and presence of cyclodextrins. From the solubility studies it was also concluded that a 1:1 molar complex was the only species formed under the conditions studied. Stability constants of 66-69 and 62-83 M^{-1} were reported for beta-cyclodextrin and DIMEB respectively.

The solid beta-cyclodextrin/salbutamol complex was prepared by freeze-drying (lyophilization), that is equimolar amounts of beta-cyclodextrin and salbutamol were dissolved in water, then subjected to freeze-drying for 48 hours while being protected from light. The formation of a solid state inclusion complex obtained from freeze-drying was determined by Differential Scanning Calorimetry. When guest molecules are incorporated in a cyclodextrin cavity their melting, boiling and / or sublimation points shift to different temperatures or disappear within the temperature range in

Samsung Bioepis Exhibit No. 1002
Page 8508

which the cyclodextrin is decomposed. The DSC thermogram for freeze dried salbutamol showed an endotherm at 431K, and the physical mix of freeze dried beta-cyclodextrin and salbutamol showed an endotherm at 423K. The thermogram of the freeze-dried complex showed no endothermic peak due to the phase transition of salbutamol. Thus the authors claim that freeze-drying resulted in a true inclusion complex being formed. However, the lack of a thermal event for the freeze dried beta-cyclodextrin/salbutamol could also indicate the amorphousness of the salbutamol in the interstitial spaces of the beta-cyclodextrin.

Cabral Marques *et al* (International Journal of Pharmaceutics, 63 (1990) 267-274) used Molecular Modelling and Proton-NMR to assess the mode of inclusion of salbutamol within the beta-CD cavity. Using Proton-NMR to assess the mode of inclusion is based on the shielding of the cyclodextrin and drug protons. If inclusion occurs, protons located within or near the cyclodextrin cavity should be strongly shielded. The NMR spectra showed upfield shifts of the cyclodextrin protons in the presence of salbutamol and the salbutamol protons shifted downfield in the presence of beta-cyclodextrin. The downfield shifts of the aromatic protons were greater than those of the aliphatic protons, suggesting that the aromatic ring of salbutamol interacts more strongly with the beta-cyclodextrin. The interior protons of the cyclodextrin molecule were shielded as a result of the anisotropy of the guest aromatic moiety. The highest shifts of beta-cyclodextrin protons occurred for a molar ratio of 1:1 (salbutamol: beta-cyclodextrin) indicating the probable stoichiometry of the complex. Molecular graphical computation showed that the minimum van der Waals energy positioning of salbutamol relative to beta-cyclodextrin occurs when the aromatic ring of salbutamol penetrates the cavity leaving the aliphatic chain externalised.

Samsung Bioepis Exhibit No. 1002
Page 8509

Cabral Marques *et al* (*International Journal of Pharmaceutics,* 77 (1991) 303-307) studied the fate of a freeze-dried 2-hydroxypropyl-ß-cyclodextrin (HP-ß-CD):salbutamol complex following pulmonary administration in order to evaluate the possibility of obtaining sustained release of salbutamol using HP-ß-CD as a carrier. Using a randomised cross-over design, the absorption and pharmacokinetics of HP-ß-CD salbutamol complex was investigated in four healthy New Zealand White male rabbits, after intravenous bolus (i.v.) by means of the marginal ear vein and pulmonary administration via intratracheal instillation (i.t.) at the bifurcation of the trachea. Although the terminal half-life of salbutamol did not change significantly after complexation, the pulmonary absorption of salbutamol as a complex was prolonged, as shown by its maximum plasma concentration which was observed approximately 23 minutes after instillation compared to approximately 14 minutes for the uncomplexed Salbutamol, and also by the increased absorption time. After i.v. dosing the availability of the complexed salbutamol was reduced to about 80% of that of the free drug. They concluded that complexation with HP-ß-CD did not sufficiently extend the salbutamol release profile to justify its use as a sustained release inhalation formulation.

Hirayama *et al* (Pharmaceutical Sciences 1995, 1, 517-520) investigated the effect of perbutanoyl-ß-cyclodextrin (TB-ß-CD), a hydrophobic cyclodextrin derivative, on plasma salbutamol levels after oral administration in dogs. The kneading method was used to prepare the sample of salbutamol/TB-ß-CD in a 1:1 molar ratio using methanol as the solvent. After oral administration of the salbutamol complex in dogs, a prolonged maintenance (at least 24 hours) of higher and constant salbutamol levels in plasma was obtained. The area under the plasma salbutamol concentration curve up to 24 hours after administration of the salbutamol/TB-ß-CD complex was about 5 times that

Samsung Bioepis Exhibit No. 1002
Page 8510

of salbutamol alone. Furthermore, the plasma level of salbutamol glucuronide, a major metabolite of salbutamol, was significantly lower after administration of the salbutamol/TB-ß-CD complex than after administration of the drug alone. This was attributed to the suppression of the metabolism of salbutamol in the gastrointestinal tract by TB-ß-CD complexation. The authors concluded that TB-ß-CD may be a useful carrier for orally administered water-soluble drugs which are extensively metabolized in the gastrointestinal tract.

Heptakis-(2,6-di-*O*-methyl)-ß-cyclodextrin (DIMEB) is derived from beta-cyclodextrin where methylation has occurred selectively at carbon 2 and 6 in each glucose subunit. Thus, there are 14 methyl groups per substituted cyclodextrin molecule. This is the beta-cyclodextrin molecule used by Cabral Marques *et al* (International Journal of Pharmaceutics, 63 (1990) 259-266). Randomly methylated-ß-cyclodextrin (RAMEB) is obtained from beta-cyclodextrin where methylation has occurred randomly at carbons 2, 3, or 6. Methylation may occur at all three positions, any two positions, or at one position only. Therefore, each glucose subunit may have one, two or three methyl groups. The degree of substitution is expressed as the average number of methyl groups per glucose subunit.

Salbutamol base and salbutamol sulphate have been complexed with various cyclodextrins in order to modify the *in vivo* release of salbutamol. Notably, salbutamol has been complexed with perbutanoyl-ß-cyclodextrin (Hirayama, F; Horikawa, T; Yamanaka, M; and Uekama, K.; *Pharmaceutical Sciences* 1995, 1:517-520) and 2-hydroxypropyl-ß-cyclodextrin (Cabral Marques, H. M.; Hadgraft, J.; Kellaway, I.W.; Taylor, G.; *International Journal of Pharmaceutics*. 77 (1991) 303-307) in order to evaluate the possibility of obtaining sustained release following oral and pulmonary administration.

Samsung Bioepis Exhibit No. 1002
Page 8511

Complexes of salbutamol base with alpha-cyclodexrin, beta-cyclodextrin, gamma-cyclodextrin and heptakis(2,6-di-O-methyl)-ß-cyclodextrin(DIMEB) were obtained in order to study the effect of temperature and cyclodextrin concentration on complex solubility (Cabral Marques, H.M.; Hadgraft, J. and Kellaway, I.W., *International Journal of Pharmaceutics*, 63 (1990) 259-266). Further, salbutamol base was complexed with beta-cyclodextrin in order that proton NMR could be employed to assess the mode of salbutamol inclusion within the beta-cyclodextrin cavity. The solid salbutamol inclusion complexes were prepared by freeze drying. Unfortunately freeze drying is an expensive drying technique and does not yield a product with acceptable physical characteristics. That is, the solid product obtained from freeze drying does not have a suitable particle size distribution, nor are the flow characteristics suitable for scale up or tabletting.

There is a need to be able to deliver $ß_2$-adrenergic agonists via the transmucosal routes.

SUMMARY OF THE INVENTION

According to a first aspect of the invention there is provided a pharmaceutical composition for oral mucosal delivery comprising as an active ingredient an inclusion complex of (a) a selective $ß_2$-adrenergic agonist or a pharmaceutically acceptable salt thereof and (b) an unsubstituted or substituted beta- or gamma-cyclodextrin, and a pharmaceutically acceptable carrier for oral mucosal delivery.

The selective $ß_2$-adrenergic agonist is preferably selected from the group consisting of salbutamol, metaproterenol, terbutaline, pirbuterol, formoterol,

Samsung Bioepis Exhibit No. 1002
Page 8512

salmeterol, fenbuterol, orciprenaline, isoprenaline, hexoprenaline, reproterol, rimiterol, fenoterol, procaterol, mabuterol, bambuterol, bitolterol, broxaterol, carbuterol, clenbuterol, coterol, eformoterol, tretoquinol and tulobuterol and the pharmaceutically acceptable salts thereof, such as for example the sulphate, methanesulfonate, hydrochloride, dihydrochloride, acetate or monoacetate salts.

The selective β_2-adrenergic agonist is preferably salbutamol either in the form of the free base or in the form of a pharmaceutically acceptable salt such as the sulphate salt.

The inclusion complex preferably has a stoichiometry of (a) to (b) of 1:1 to 1:10 mol/mol, more preferably 1:1 to 1:5 mol/mol, most preferably 1:1 mol/mol.

The pharmaceutical composition may be formulated in the form of a buccal or sublingual tablet, lozenge, adhesive patch, gel, solution or spray.

The pharmaceutical composition is preferably for use in the treatment of a reversible obstructive airways disease such as asthma or the reversible component of chronic obstructive pulmonary diseases.

According to a second aspect of the invention there is provided the use of an inclusion complex of (a) a selective β_2-adrenergic agonist or a pharmaceutically acceptable salt thereof and (b) an unsubstituted or substituted beta- or gamma-cyclodextrin, in the manufacture of a pharmaceutical composition for oral mucosal delivery for use in a method of treatment of a reversible obstructive airways disease such as asthma or the reversible component of chronic obstructive pulmonary diseases.

Samsung Bioepis Exhibit No. 1002
Page 8513

According to a third aspect of the invention there is provided a method of treating a patient suffering from a reversible obstructive airways disease which comprises administering to the oral mucosae of the patient an inclusion complex of (a) a selective $ß_2$-adrenergic agonist or a pharmaceutically acceptable salt thereof and (b) an unsubstituted or substituted beta- or gamma-cyclodextrin.

According to a fourth aspect of the invention there is provided an inclusion complex of salbutamol base and randomly substituted methyl-beta-cyclodextrin which has substantially the X-ray powder diffraction pattern of Figure 1 of the accompanying drawings or the Fourier Transform Infra-red spectrum of Figure 2 of the accompanying drawings.

According to a fifth aspect of the invention there is provided an inclusion complex of salbutamol base and 2-hydroxypropyl-beta-cyclodextrin which has substantially the X-ray powder diffraction pattern of Figure 5 of the accompanying drawings or the Fourier Transform Infra-red spectrum of Figure 6 of the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows an X-ray powder diffraction pattern of a 1:1 kneaded complex of salbutamol base and randomly methylated-ß-cyclodextrin obtained from Example 1;

Figure 2 shows a Fourier Transform infra-red spectrum of a 1:1 kneaded complex of salbutamol base and randomly methylated-ß-cyclodextrin obtained from Example 1;

Samsung Bioepis Exhibit No. 1002
Page 8514

Figure 3 shows a Differential Scanning Calorimetry thermogram of salbutamol base with a onset melting temperature of 157°C and a sharp endothermic melting peak at 159°C;

Figure 4 shows a Differential Scanning Calorimetry thermogram of a 1:1 kneaded complex of salbutamol base and randomly methylated-ß-cyclodextrin obtained from Example 1;

Figure 5 shows an X-ray powder diffraction pattern of an 1:1 kneaded complex of salbutamol base and 2-hydroxypropyl-ß-cyclodextrin obtained from Example 2;

Figure 6 shows a Fourier Transform infra-red spectrum of a 1:1 kneaded complex of salbutamol base and 2-hydroxypropyl-ß-cyclodextrin obtained from Example 2; and

Figure 7 shows a Differential Scanning Calorimetry thermogram a 1:1 kneaded complex of salbutamol base and 2-hydroxypropyl-ß-cyclodextrin obtained from Example 2.

DESCRIPTION OF EMBODIMENTS

The crux of the invention is a pharmaceutical composition for oral mucosal delivery which comprises as an active ingredient an inclusion complex of (a) a selective $ß_2$-adrenergic agonist or a pharmaceutically acceptable salt thereof and (b) an unsubstituted beta- or gamma-cyclodextrin, together with a pharmaceutically acceptable carrier for oral mucosal delivery.

Samsung Bioepis Exhibit No. 1002
Page 8515

Examples of suitable compounds (a) are salbutamol, metaproterenol, terbutaline, pirbuterol, formoterol, salmeterol, fenbuterol, orciprenaline, isoprenaline, hexoprenaline, reproterol, rimiterol, fenoterol, procaterol, mabuterol, bambuterol, bitolterol, broxaterol, carbuterol, clenbuterol, coterol, eformoterol, tretoquinol and tulobuterol. The compound may be used in the form of the free base, or in the form of a pharmaceutically acceptable salt such as a sulfate, methanesulfonate, hydrochloride, dihydrochloride, or monoacetate salt.

The second component of the inclusion complex is an unsubstituted or substituted beta- or gamma-cyclodextrin.

Highly water soluble cyclodextrins such as 2-hydroxypropylated or methylated or sulphoalkylated derivatives of beta-cyclodextrin are the preferred cyclodextrins of the invention. Gamma-cyclodextrin or 2-hydroxypropylated or methylated or sulphoalkylated derivatives of gamma-cyclodextrin may also be used in the same manner as the corresponding preferred beta-cyclodextrin derivatives. The degree of substitution of the cyclodextrin derivatives may vary between 1 to 20 substituents per cyclodextrin molecule but more preferably between 3 to 15 substituents per cyclodextrin molecule. When the cyclodextrin is 2-hydroxypropyl-beta-cyclodextrin, the preferred degree of substitution is between 3,9 and 5,1 hydroxypropyl groups per cyclodextrin molecule. When the cyclodextrin is randomly methylated-beta-cyclodextrin, the preferred degree of substitution is between 1,8 and 2 methyl groups per glucose unit.

The inclusion complex may be prepared from aqueous solutions, slurries or pastes of the drug active and cyclodextrin according to conventional methods. The molar ratio of drug active to cyclodextrin may vary between

Samsung Bioepis Exhibit No. 1002
Page 8516

1:1 and 1:10, but more preferably between 1:1 to 1:5 and most preferably about 1:1. Solutions are prepared by dissolving the cyclodextrin in a sufficient quantity of purified, deionized water which may be optionally buffered between pH 7,4 and 8,5. The drug active is added to the solution with stirring until dissolved. The solution may be dried by spray drying, freeze drying or by evaporation of the aqueous phase under reduced pressure and/ or increased temperature. Alternatively, the drug active and cyclodextrin are mixed in the dry form. The powder mixture is wetted with water, optionally containing a buffer, pH 7,4 - 8,5, while mixing vigorously until a paste or slurry is formed. The paste or slurry is mixed for 0,25 to 2 hours and is then dried in an oven or *in vacuo* at elevated temperature. The dried complex obtained may be crushed or milled and sieved to the desired particle size.

A pharmaceutically acceptable buffer, capable of buffering in the pH range 7,4-8,5 may be used in the formation of the inclusion complex, particularly when the drug active is present as a salt. Preferred buffers include tromethamine, triethanolamine, diethanolamine and phosphate buffer. The concentration of the buffer may vary from 0,1 to 5 molar equivalents relative to the drug active.

The administration of a selective $ß_2$-adrenergic agonist through the mucosal tissue of the mouth avoids the problems associated with administration of these drugs by inhalation (i.e. only approximately 10% of the drug entering the lungs, difficulty with inhalation techniques resulting in therapeutic blood levels not being attained) and by oral administration (i.e. slower onset of action and extensive metabolism in the gastrointestinal tract).

Absorption of the drug from the pharmaceutical composition of the invention

Samsung Bioepis Exhibit No. 1002
Page 8517

through the oral mucosae is rapid such that the drug reaches the systemic circulation within minutes, which is highly advantageous for the rapid relief of bronchoconstriction associated with a reversible obstructive airways disease such as asthma or the reversible component of chronic obstructive pulmonary diseases. Further, any unpleasant taste and irritant properties of the active are reduced by presenting the drug to the oral mucosal membranes in the form of a cyclodextrin inclusion complex.

The present invention achieves these advantages by molecular encapsulation of the selective β_2-adrenergic agonist in a cyclodextrin molecule, so forming a molecular inclusion complex which may be used in the solid form for the preparation of a pharmaceutical composition for oral mucosal delivery, such as buccal or sublingual tablets, lozenges, adhesive patches, gels, solutions and sprays.

Administration of a single dose of a sublingual tablet containing 4mg salbutamol according to the invention resulted in relief of asthma within 2 minutes in two asthmatic patients.

The complex according to the invention may be incorporated into a shearform matrix designed for immediate release as described in Fuisz Technologies Ltd patents (Eur. Pat. Appl. EP 95-650038 and PCT Int. Appl. WO 95/34293).

The pharmaceutical composition of the invention may also contain, in addition to the pharmaceutically acceptable carrier for oral mucosal delivery, other optional ingredients as are discussed below.

Penetration enhancers may be used to promote the passage of the drug

Samsung Bioepis Exhibit No. 1002
Page 8518

active across the mucosal membranes. Typical permeation enhancers include fatty acids and their salts such as sodium caprate, sodium caprylate and sodium oleate, and bile salts such as sodium glycodeoxycholate, sodium glycocholate, sodium cholate and sodium taurodeoxycholate. Other penetration enhancers may include tensides or non-ionic surfactants such as polyethylene glycol 660 hydroxystearate or polyoxyethylene lauryl ethers, fusidates such as sodium taurodihydrofusidate, azone and chitosan. Combinations of permeation enhancers such as polyoxyethylene 8 lauryl ether and sodium glycocholate or mixed micelles such as sodium caprate and sodium glycocholate may also be used. The penetration enhancers may also be used in combination with beta- or gamma-cyclodextrins or their methyl, hydroxypropyl or sulphoalkyl derivatives. Typical concentrations of penetration enhancers are between 0,1 and 5,0%, more preferably between 0,25 to 3% by mass of the inclusion complex.

As stated above, the selective β_2-adrenergic agonist may be used in the form of the free base or a pharmaceutically acceptable salt. When acidic penetration enhancing excipients are used such as bile acids or fatty acids, or pharmaceutically acceptable salts of bile acids, salt formation between the basic component of the β_2-adrenergic agonist and the acidic component of the bile or fatty acid may occur.

Buffering agents may be incorporated into the pharmaceutical composition of the invention to control the microenvironmental pH surrounding the drug delivery system in the alkaline range, so as to maximise the percentage of the unionized form of the drug, as drugs in the unionized form cross mucosal membranes more readily than the corresponding ionized form.

The pharmaceutical composition may contain suitable flavouring and

Samsung Bioepis Exhibit No. 1002
Page 8519

sweetening agents such as mint, spearmint, aspartame, sucrose, xylitol, saccharin and the like.

Typical sublingual or buccal tablets may include lubricants such as magnesium stearate, calcium stearate and sodium stearyl fumarate to facilitate tablet compression. Diluents such as lactose, microcrystalline cellulose, maize starch and the like may be used. In order to prevent movement of the dosage form from the site of administration, mucoadhesive agents such as chitosan, carbopol 934P, and hydroxypropyl cellulose and the like may be used in conventional concentrations.

Typical disintegrants to enhance sublingual or buccal tablet disintegration include sodium carboxymethylcellulose, sodium starch glycolate, polyplasdone XL, and dried starch.

Another aspect of the invention is the specific inclusion complex of salbutamol base and methyl-beta-cyclodextrin having substantially the X-ray powder diffraction pattern of Figure 1 or the Fourier Transform Infra-red spectrum of Figure 2 of the accompanying drawings.

A further aspect of the invention is the specific inclusion complex of salbutamol base and 2-hydroxypropyl-ß-cyclodextrin having substantially the X-ray powder diffraction pattern of Figure 5 or the Fourier Transform Infra-red spectrum of Figure 6 of the accompanying drawings.

The following examples illustrate the present invention.

Example 1

Samsung Bioepis Exhibit No. 1002
Page 8520

Salbutamol base (2,00g) and methyl-beta-cyclodextrin (11,57g) are mixed together in a mortar. Purified, deionized water (6ml) is added in aliquots with mixing to form a homogenous paste. The paste is dried in an oven at 40°C and atmospheric pressure. The dried complex is crushed with a pestle and passed through a 250µm mesh sieve. At all processing stages, the compound is protected from light. The complex contains 14,1% mass/mass salbutamol base as determined by HPLC.

Example 2

Salbutamol base (2,00g) and 2-hydroxypropyl-beta-cyclodextrin (12,6g) are mixed together in a mortar. Purified, deionized water (10ml) is added in aliquots with mixing to form a homogenous paste. Continue grinding for 0,5 hours. The paste is dried in an oven at 40°C and 5 millibar. The dried complex is crushed with a pestle and passed through a 250µm mesh sieve. At all processing stages, the compound is protected from light. The complex contains 13,24% mass/mass salbutamol base as determined by HPLC.

Example 3

The unit composition of a sublingual tablet containing the equivalent of 4 mg salbutamol base is as follows:

Salbutamol base/methyl-beta-cyclodextrin complex (from Example 1)	28,5mg
Xylitol	21,0 mg
Sodium Stearyl Fumarate	0,5 mg

The complex is blended with the xylitol. The lubricant, sodium stearyl fumarate, is screened in and the mixture is blended and formed into sublingual tablets by compression at 10 - 30 N.

Samsung Bioepis Exhibit No. 1002
Page 8521

Example 4

The unit composition of a sublingual tablet containing the equivalent of 4 mg salbutamol base is as follows:

Salbutamol base/2-hydroxypropyl-ß-CD (from Example 2)	30,2 mg
Microcrystalline Cellulose	8,6 mg
Sodium Starch Glycollate	10,0 mg
Chitosan	0,6 mg
Magnesium Stearate	0,6 mg

The complex is blended with the microcrystalline cellulose, sodium starch glycollate and chitosan. The lubricant, magnesium stearate, is screened in and the mixture is blended and formed into sublingual tablets by compression at 10 - 30 N.

Example 5

The unit composition of a sublingual tablet containing the equivalent of 4 mg salbutamol base is as follows.

Salbutomol sulphate/2-hydroxypropyl-ß-CD	30,8 mg
Tromethamine	25,0 mg
Microcrystalline cellulose	8,2 mg
Sodium starch glycollate	10,0 mg
Xylitol	25,3 mg
Menthol	0,1 mg
Mangnesium stearate	0,6 mg

Example 6

Tulobuterol base (10,0 g) and randomly methylated beta-cyclodextrin (57,0 g) are mixed together in a mortar. Purified, deionised water (30 mL)

Samsung Bioepis Exhibit No. 1002
Page 8522

is added in aliquots with mixing to form a homogeneous paste. Grinding is continued for a further 1 hour. The paste is dried in an oven at 50°C and 5 millibar. The dried complex is crushed with a pestle and passed through a 250μm mesh sieve. The complex contains 14,93% mass/mass tulobuterol base as determined by HPLC.

Example 7

The unit composition of a sublingual tablet containing the equivalent of 2 mg tulobuterol base is as follows:

Tulobuterol base/randomly methylated beta-cyclodextrin	13,40 mg
Xylitol	43,10 mg
Anhydrous Lactose	43,00 mg
Sodium stearyl fumarate	0,50 mg

The complex is blended with the xylitol and anhydrous lactose. The lubricant, sodium stearyl fumarate is screened in and the mixture is blended and formed into sublingual tablets by compression to 10-30N.

Inclusion complexes of the other β_2-adrenergic agonists listed above and any suitable beta- or gamma-cyclodextrin can be manufactured in the same manner as exemplified for salbutamol and tulobuterol and can be formulated in the same manner as exemplified for salbutamol and tulobuterol.

The procedures used to obtain the results shown in Figures 1 to 7 are as follows:

X-ray powder diffraction patterns were obtained using a Rigaku D Max 3 powder diffractometer with a graphite monochromator and Cu radiation. Samples were mounted in Al rings, or on glass slides and run from 2° -

Samsung Bioepis Exhibit No. 1002
Page 8523

52° 2θ, with a step size of 0.02°θ and a fixed time of 1s.

Fourier Transform infra-red transmission spectra were obtaining using a Perkin Elmer Spectrum 2000 spectrometer. Data was collected from 4400 cm^{-1} to 600 cm^{-1} with a resolution of 4 cm^{-1}, strong apodization and a gain of 1. Samples were prepared as powders in KBr matrix and data was collected from diffuse reflectance cells.

Differential scanning calorimetry thermograms were obtained using Perkin Elmer DSC 7 Differential Scanning Calorimeter. Sampling was done in vented aluminium pans (50µl). The starting temperature was 50°C, final temperature was 200°C, and the rate of temperature increase was 10°C/minute. The carrier gas was nitrogen. The instrument was calibrated with an indium standard prior to the sample being analysed.

The treatment or prevention of bronchospasm with selective β_2-adrenergic agonists via the oral transmucosal route has the following advantages over currently available treatments:

1 Less complicated treatment modality than inhalation, particularly when used during an attack and especially significant for pediatric use.

2 Better compliance than inhalers in cases where the presence of excessive secretions within the airway lumen or thickening of the airway epithelium (due to oedema, infiltration of the mucosa by eosinophils and other inflammatory cells, hypertrophy and hyperplasia) results in reduced access of the inhaled drug to the site of action.

3 Similar time of onset of action when compared with inhalers and significantly faster than that of orogastric tablets or syrup.

4 Potential for 50% dose reduction relative to tablet or syrup dose due to avoidance of presystemic hepatic metabolism.

Samsung Bioepis Exhibit No. 1002
Page 8524

5 More convenient than inhalers for use in public.

6 More convenient than inhalers for prevention of exercise-induced brochospasm, where the drug should be administered 15 minutes before exercise.

Samsung Bioepis Exhibit No. 1002
Page 8525

CLAIMS

1 A pharmaceutical composition for oral mucosal delivery comprising as an active ingredient an inclusion complex of (a) a selective $ß_2$-adrenergic agonist or a pharmaceutically acceptable salt thereof and (b) an unsubstituted or substituted beta- or gamma-cyclodextrin, and a pharmaceutically acceptable carrier for oral mucosal delivery.

2 A pharmaceutical composition according to claim 1 wherein the selective $ß_2$-adrenergic agonist is selected from the group consisting of salbutamol, metaproterenol, terbutaline, pirbuterol, formoterol, salmeterol, fenbuterol, orciprenaline, isoprenaline, hexoprenaline, reproterol, rimiterol, fenoterol, procaterol, mabuterol, bambuterol, bitolterol, broxaterol, carbuterol, clenbuterol, coterol, eformoterol, tretoquinol and tulobuterol and the pharmaceutically acceptable salts thereof.

3 A pharmaceutical composition according to claim 1 or claim 2 wherein the selective $ß_2$-adrenergic agonist is salbutamol in the form of the free base or in the form of a pharmaceutically acceptable salt.

4 A pharmaceutical composition according to any one of claims 1 to 3 wherein the cyclodextrin is selected from the group consisting of unsubstituted beta-cyclodextrin, 2-hydroxypropylated beta-cyclodextrin, methylated beta-cyclodextrin and sulphoalkylated beta-cyclodextrin.

5 A pharmaceutical composition according to any one of claims 1 to 3 wherein the cyclodextrin is selected from the group consisting of unsubstituted gamma-cyclodextrin, 2-hydroxypropylated gamma-

Samsung Bioepis Exhibit No. 1002
Page 8526

cyclodextrin, methylated gamma-cyclodextrin and sulphoalkylated gamma-cyclodextrin.

6 A pharmaceutical composition according to any one of claims 1 to 5 wherein the inclusion complex has a stoichiometry of (a) to (b) of 1:1 to 1:10 mol/mol.

7 A pharmaceutical composition according to claim 6 wherein the inclusion complex has a stoichiometry of (a) to (b) of 1:1 to 1:5 mol/mol.

8 A pharmaceutical composition according to claim 7 wherein the inclusion complex has a stoichiometry of (a) to (b) of 1:1 mol/mol.

9 A pharmaceutical composition according to any one of claims 1 to 8 formulated in a form selected from the group consisting of a buccal tablet, a sublingual tablet, a lozenge, an adhesive patch, a gel, a solution and a spray.

10 A pharmaceutical composition according to any one of claims 1 to 9 for use in the treatment of a reversible obstructive airways disease.

11 The use of an inclusion complex of (a) a selective β_2-adrenergic agonist or a pharmaceutically acceptable salt thereof and (b) an unsubstituted or substituted beta- or gamma-cyclodextrin, in the manufacture of a pharmaceutical composition for oral mucosal delivery for use in a method of treatment of a reversible obstructive airways disease.

12 The use according to claim 11 wherein the reversible obstructive

Samsung Bioepis Exhibit No. 1002
Page 8527

airways disease is asthma or the reversible component of chronic obstructive pulmonary diseases.

13 The use according to claim 11 or claim 12 wherein the selective $ß_2$-adrenergic agonist is selected from the group consisting of salbutamol, metaproterenol, terbutaline, pirbuterol, formoterol, salmeterol, fenbuterol, orciprenaline, isoprenaline, hexoprenaline, reproterol, rimiterol, fenoterol, procaterol, mabuterol, bambuterol, bitolterol, broxaterol, carbuterol, clenbuterol, coterol, eformoterol, tretoquinol and tulobuterol and the pharmaceutically acceptable salts thereof.

14 The use according to claim 13 wherein the selective $ß_2$-adrenergic agonist is salbutamol in the form of the free base or in the form of a pharmaceutically acceptable salt.

15 The use according to any one of claims 11 to 14 wherein the cyclodextrin is selected from the group consisting of unsubstituted beta-cyclodextrin, 2-hydroxypropylated beta-cyclodextrin, methylated beta-cyclodextrin, and sulphoalkylated beta-cyclodextrin.

16 The use according to any one of claims 1 to 14 wherein the cyclodextrin is selected from the group consisting of unsubstituted gamma-cyclodextrin, 2-hydroxypropylated gamma-cyclodextrin, methylated gamma-cyclodextrin and sulphoalkylated gamma-cyclodextrin.

17 The use according to any one of claims 1 to 16 wherein the inclusion complex has a stoichiometry of (a) to (b) of 1:1 to 1:10 mol/mol.

Samsung Bioepis Exhibit No. 1002
Page 8528

18 The use according to claim 17 wherein the inclusion complex has a stoichiometry of (a) to (b) of 1:1 to 1:5 mol/mol.

19 The use according to claim 18 wherein the inclusion complex has a stoichiometry of (a) to (b) of 1:1 mol/mol.

20 The use according to any one of claims 11 to 19 wherein the pharmaceutical composition is selected from the group consisting of a buccal tablet, a sublingual tablet, a lozenge, an adhesive patch, a gel, a solution or a spray.

21 A method of treating a patient suffering from a reversible obstructive airways disease comprising administering to the oral mucosae of the patient an inclusion complex of (a) a selective $ß_2$-adrenergic agonist or a pharmaceutically acceptable salt thereof and (b) an unsubstituted or substituted beta- or gamma-cyclodextrin.

22 A method according to claim 21 wherein the reversible obstructive airways disease is asthma or the reversible component of chronic obstructive pulmonary diseases.

23 An inclusion complex of salbutamol base and randomly substituted methyl-beta-cyclodextrin which has substantially the X-ray powder diffraction pattern of Figure 1 of the accompanying drawings or the Fourier Transform Infra-red spectrum of Figure 2 of the accompanying drawings.

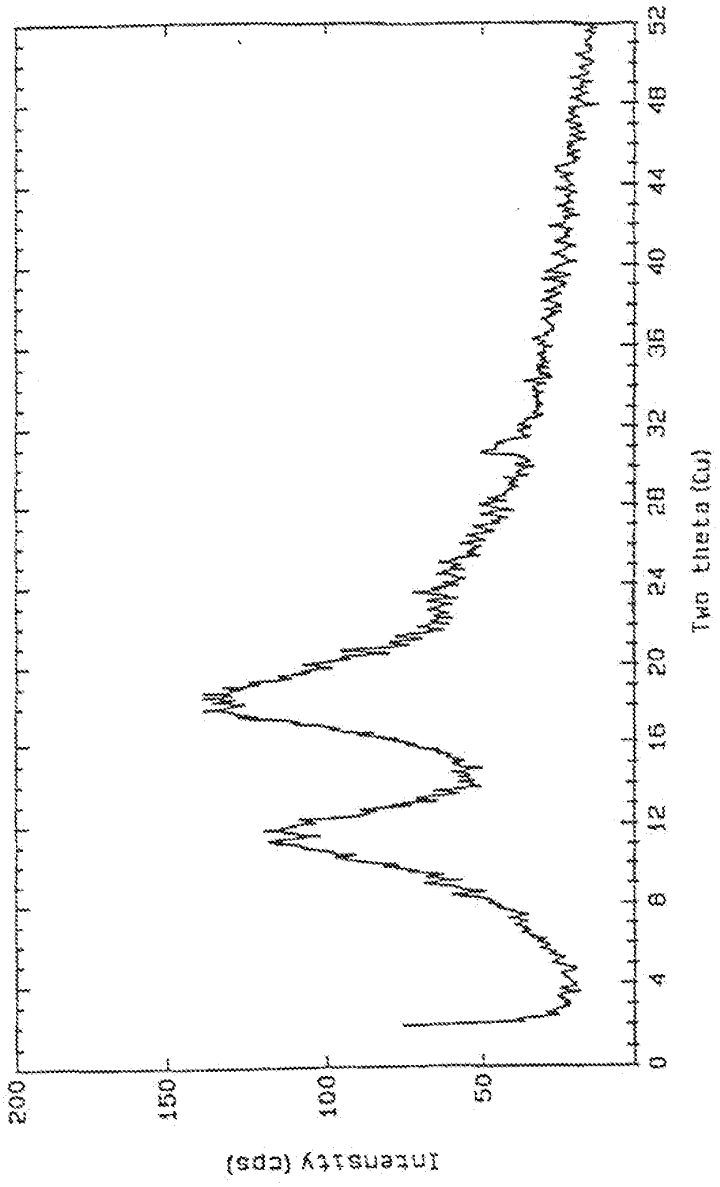
24 An inclusion complex of salbutamol base and 2-hydroxypropyl-beta-cyclodextrin which has substantially the X-ray powder diffraction

Samsung Bioepis Exhibit No. 1002
Page 8529

pattern of Figure 5 of the accompanying drawings or the Fourier Transform Infra-red spectrum of Figure 6 of the accompanying drawings.

Samsung Bioepis Exhibit No. 1002
Page 8530

FIGURE 1



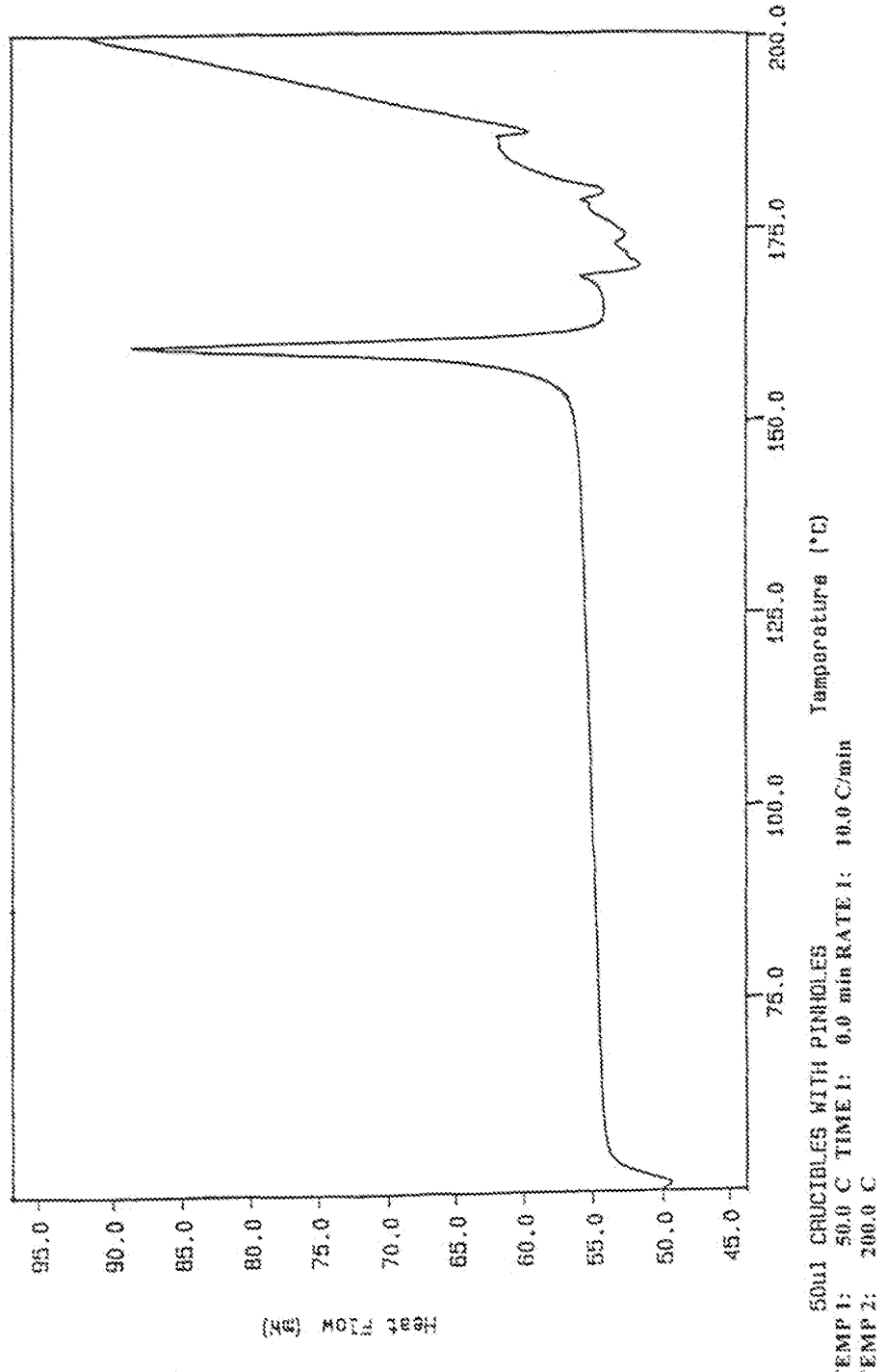
Samsung Bioepis Exhibit No. 1002
Page 8531

FIGURE 2

SUBSTITUTE SHEET (RULE 26)
401

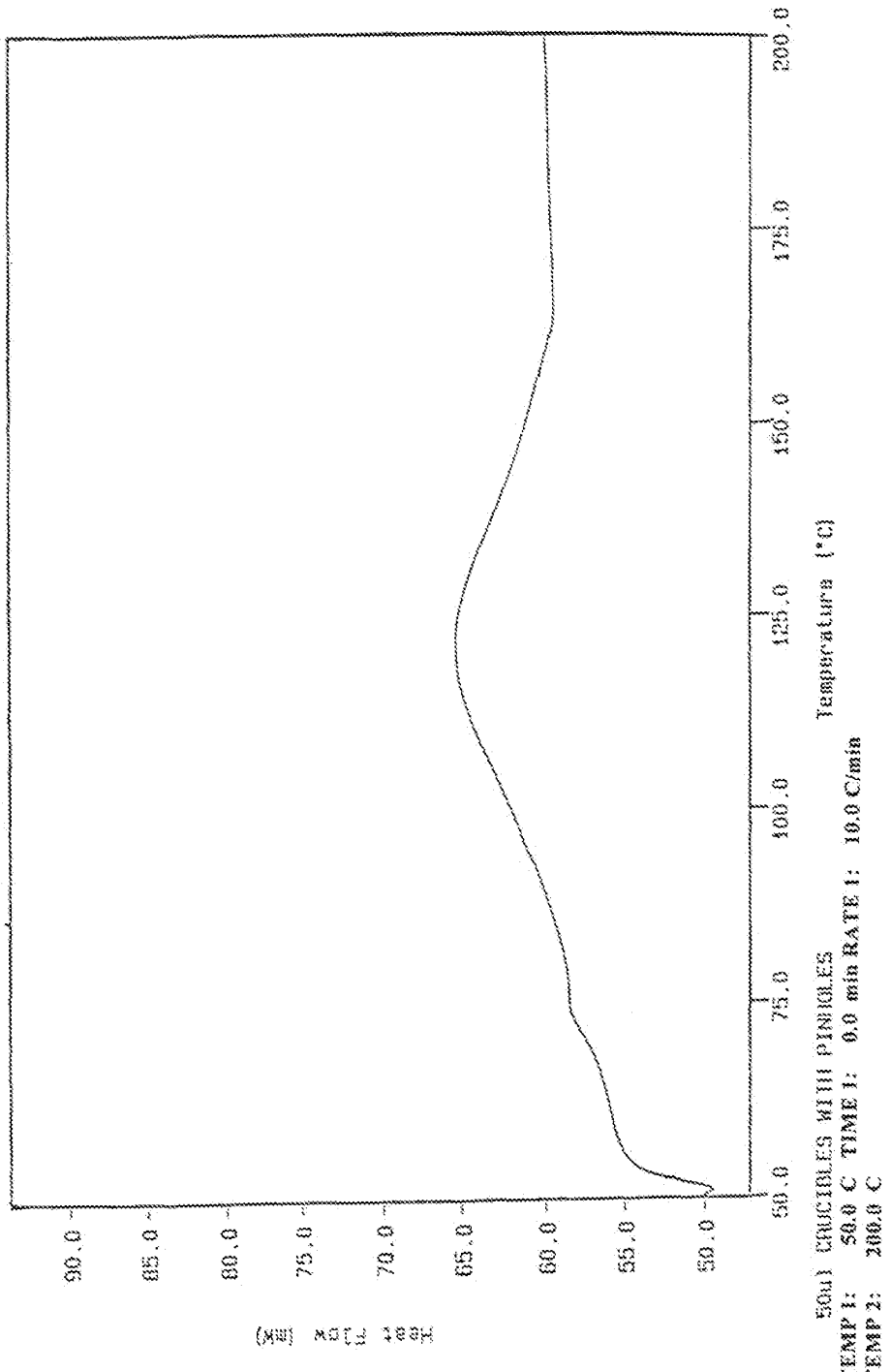
Samsung Bioepis Exhibit No. 1002
Page 8532

FIGURE 3



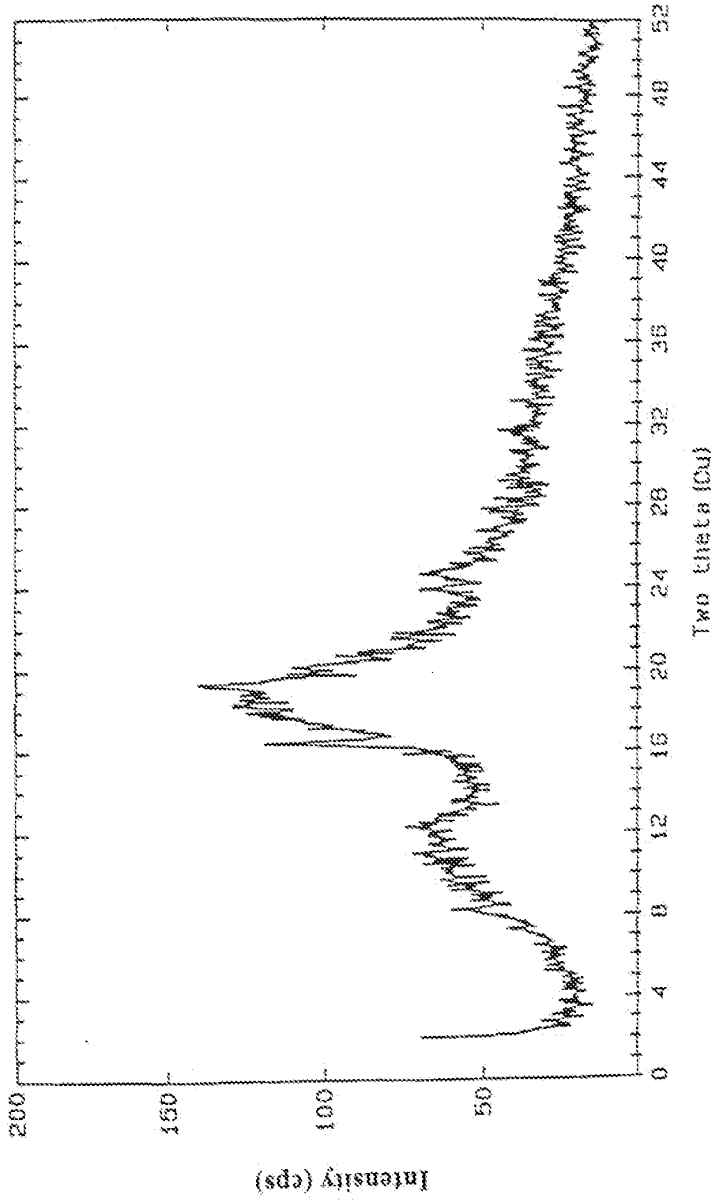
Samsung Bioepis Exhibit No. 1002
Page 8533

FIGURE 4



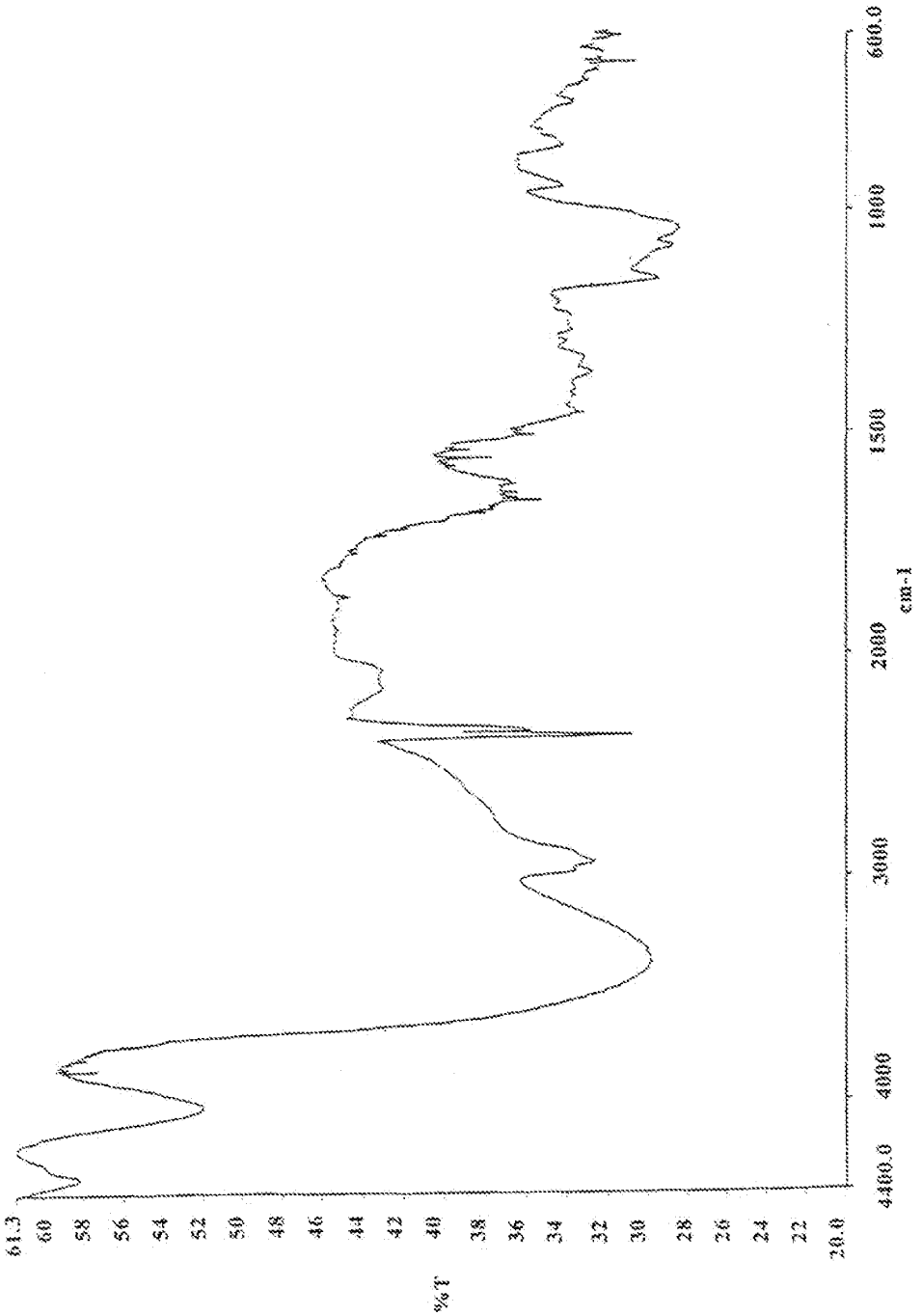
Samsung Bioepis Exhibit No. 1002
Page 8534

FIGURE 5



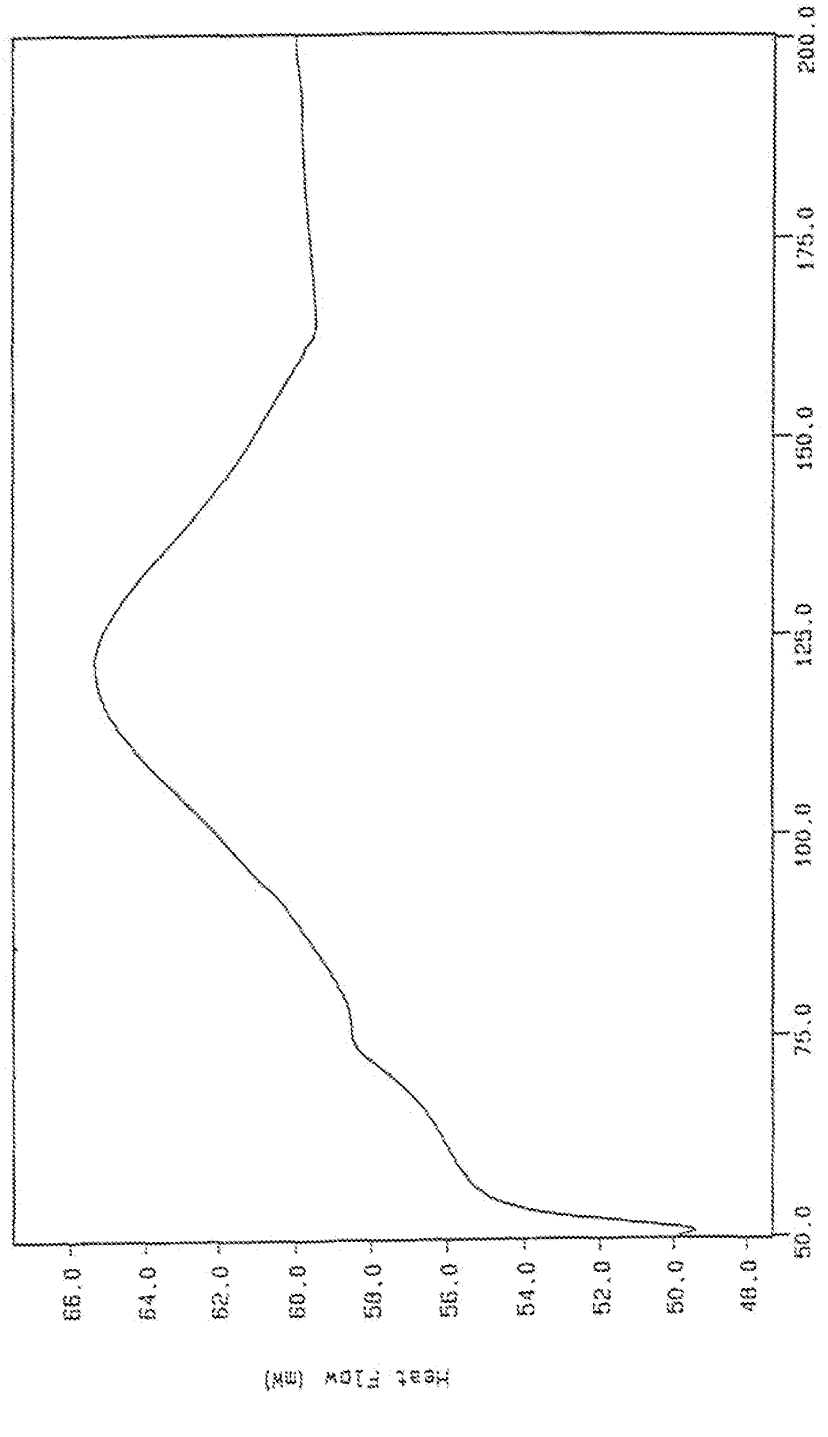
Samsung Bioepis Exhibit No. 1002
Page 8535

FIGURE 6



Samsung Bioepis Exhibit No. 1002
Page 8536

FIGURE 7



Samsung Bioepis Exhibit No. 1002
Page 8537

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02947

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C08B37/00 A61K47/48 A61K31/135

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C08B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5 080 903 A (AYACHE JOSIANE ET AL) 14 January 1992 * see in particular claims 1-4; col. 2, lines 65-68 *	1-24
Y	WO 96 01129 A (FARMARC NEDERLAND BV ;PENKLER LAWRENCE JOHN (ZA); GLINTENKAMP LUET) 18 January 1996 * see p.13, l.3-18, preparation examples 7 & 8;claims 1,10 *	1-24
A	EP 0 251 459 A (EURO CELTIQUE SA) 7 January 1988 * see claims 1,2; p. 3, l. 15-25 & 40 *	1-22

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 February 1998	20.02.98

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Isert, B

Form PCT/ISA/210 (second sheet) (July 1992)

Samsung Bioepis Exhibit No. 1002
Page 8538

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 97/02947

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LEMESLE-LAMACHE: "Study of beta-cyclodextrin and ethylated beta-cyclodextrin salbutamol complexes, in vitro evaluation of sustained release bahaviour of salbutamol" INT. J. PHARM., vol. 141, no. 1,2, 1996, pages 117-124, XP002054595 * see the abstract *	1-24
A	MARQUES ET AL.: "Studies of cyclodextrin inclusion complexes. I The salbutamol-cyclodextrin comple as studied by phase solubility and DSC " INT. J. PHARM., vol. 63, 1990, pages 259-266, XP002054596 cited in the application * see the abstract; p.265, conclusion *	1-24
Y	" * "	23
A	MARQUES ET AL.: "Studies of cyclodextrin inclusion complexes. IV. The pulmonary absorption of salbutamol from a complex with 2-hydroxypropyl-beta-cyclodextrin in rabbits" INT. J. PHARM., vol. 77, 1991, pages 303-307, XP002054597 cited in the application * see the abstract; p.307, conclusion *	1-24
Y	* " *	24
Y	DARROUZET H: "PREPARING CYCLODEXTRIN INCLUSION COMPOUNDS" MANUFACTURING CHEMIST, vol. 64, no. 11, 1 November 1993, page 33/34 XP000423501 * see the whole document *	23,24

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

Samsung Bioepis Exhibit No. 1002
Page 8539

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 97/02947

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5080903 A	14-01-92	FR 2619718 A	03-03-89
		AT 138565 T	15-06-96
		CA 1328621 A	19-04-94
		DE 3855323 D	04-07-96
		DE 3855323 T	10-10-96
		EP 0306422 A	08-03-89
		ES 2087855 T	01-08-96
		JP 1100117 A	18-04-89
WO 9601129 A	18-01-96	AU 3075395 A	25-01-96
		EP 0768897 A	23-04-97
		ZA 9505582 A	26-02-96
EP 0251459 A	07-01-88	DE 3780223 A	13-08-92
		JP 2052930 C	10-05-96
		JP 7080788 B	30-08-95
		JP 62292733 A	19-12-87
		US 4834985 A	30-05-89

Electronic Acknowledgement Receipt	
EFS ID:	6810430
Application Number:	11127438
International Application Number:	
Confirmation Number:	5259
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Customer Number:	28120
Filer:	Ryan David Murphey/Andrea Silverman
Filer Authorized By:	Ryan David Murphey
Attorney Docket Number:	ALXN-P02-102
Receipt Date:	14-JAN-2010
Filing Date:	11-MAY-2005
Time Stamp:	14:18:21
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	SIDS.pdf	73060 dfc2a4b6173a11ff235013576a99a4d871bfbad9	no	2
Warnings:					
Information:					

Samsung Bioepis Exhibit No. 1002
Page 8541

2	Information Disclosure Statement (IDS) Filed (SB/08)	SB08.pdf	110896 701f4891e7866c509082d6319dbd0a9838eca26c	no	1
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Foreign Reference	WO9818827.pdf	4656047 3ffef1a7fc6ea055192bbeb83f780bbd98b0011b	no	41
Warnings:					
Information:					
		Total Files Size (in bytes):	4840003		

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111
If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Samsung Bioepis Exhibit No. 1002
Page 8542

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Wang et al.

Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES Examiner: F. P. Vandervegt

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

MS Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

Pursuant to 37 CFR 1.56, 1.97 and 1.98, the attention of the Patent and Trademark Office is hereby directed to the reference listed on the attached PTO/SB/08. It is respectfully requested that the information be expressly considered during the prosecution of this application, and that the reference be made of record therein and appear among the "References Cited" on any patent to issue therefrom.

This Information Disclosure Statement is filed before the mailing date of a first Office Action after the filing of a Request for Continued Examination under 37 CFR 1.114 (37 CFR 1.97(b)(4)).

Applicants submit herewith copies of a foreign patent in accordance with 37 CFR 1.98(a)(2).

In accordance with 37 CFR 1.97(g), the filing of this Information Disclosure Statement shall not be construed to mean that a search has been made or that no other material information as defined in 37 CFR 1.56(a) exists. In accordance with 37 CFR 1.97(h), the filing of this Information Disclosure Statement shall not be construed to be an admission that any patent, publication or other information referred to therein is "prior art" for this invention unless specifically designated as such.

It is submitted that the Information Disclosure Statement is in compliance with 37 CFR 1.98 and the Examiner is respectfully requested to consider the listed reference.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: January 14, 2010

Respectfully submitted,

By /Ryan Murphey/
Ryan Murphey, Ph.D.
Registration No.: 61,156
ROPES & GRAY LLP
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Boston, Massachusetts 02110
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(617) 951-7050 (Fax)
Attorneys/Agents For Applicants

UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
11/127,438	05/11/2005	Yi Wang	ALXN-P02-102	5259

28120 7590 08/17/2010

ROPES & GRAY LLP
PATENT DOCKETING 39/41
ONE INTERNATIONAL PLACE
BOSTON, MA 02110-2624

EXAMINER	
GAMBEL, PHILLIP	

ART UNIT	PAPER NUMBER
1644	

MAIL DATE	DELIVERY MODE
08/17/2010	PAPER

Please find below and/or attached an Office communication concerning this application or proceeding.

The time period for reply, if any, is set in the attached communication.

Office Action Summary	Application No.	Applicant(s)	
	11/127,438	WANG, YI	
	Examiner	Art Unit	
	Phillip Gambel	1644	

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

Status

1)☒ Responsive to communication(s) filed on *21 September 2009*.

2a)☐ This action is **FINAL**. 2b)☒ This action is non-final.

3)☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

Disposition of Claims

4)☒ Claim(s) *15-45* is/are pending in the application.

4a) Of the above claim(s) *31-35* is/are withdrawn from consideration.

5)☐ Claim(s) _____ is/are allowed.

6)☒ Claim(s) *15-30* is/are rejected.

7)☐ Claim(s) _____ is/are objected to.

8)☐ Claim(s) _____ are subject to restriction and/or election requirement.

Application Papers

9)☐ The specification is objected to by the Examiner.

10)☐ The drawing(s) filed on _____ is/are: a)☐ accepted or b)☐ objected to by the Examiner.

Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).

Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

11)☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

Priority under 35 U.S.C. § 119

12)☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

a)☐ All b)☐ Some * c)☐ None of:

1.☐ Certified copies of the priority documents have been received.

2.☐ Certified copies of the priority documents have been received in Application No. _____.

3.☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

* See the attached detailed Office action for a list of the certified copies not received.

Attachment(s)

1) ☒ Notice of References Cited (PTO-892)
2) ☐ Notice of Draftsperson's Patent Drawing Review (PTO-948)
3) ☒ Information Disclosure Statement(s) (PTO/SB/08) Paper No(s)/Mail Date *01/24/2010*.
4) ☐ Interview Summary (PTO-413) Paper No(s)/Mail Date. _____ .
5) ☐ Notice of Informal Patent Application
6) ☐ Other: _____.

DETAILED ACTION

1. The examiner of your application in the PTO has changed. To aid in correlating any papers for this application, all further correspondence regarding this application should be directed to Technology Center 1600, Art Unit 1644.

2. A request for continued examination under 37 CFR 1.114, including the fee set forth in 37 CFR 1.17(e), was filed in this application after final rejection. Since this application is eligible for continued examination under 37 CFR 1.114, and the fee set forth in 37 CFR 1.17(e) has been timely paid, the finality of the previous Office action has been withdrawn pursuant to 37 CFR 1.114. Applicant's submission, filed on 09/21/2009, has been entered.

3. Applicant's amendment, filed 09/21/2009, has been entered.

Claim 24 has been amended.

Claims 31-45 have been added.

Claims 1-14 have been canceled previously.

Claims 15-45 are pending.

Newly submitted claims 31-45 are directed to an invention that is independent or distinct from the invention originally claimed for the following reasons: Newly submitted claims 31-45 are drawn to methods of treating pulmonary conditions with the aerosol composition of the elected invention. The method claims are related as products and processes of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case, the antibody in the aerosol composition can be used for affinity purification or immunodetection as well as other methods of treatment. Alternatively, methods of treating pulmonary conditions can be accomplished by numerous pharmaceutical agents other than anti-C5 antibodies. Therefore, they are patentably distinct.

Since applicant has received an action on the merits for the originally presented invention, this invention has been constructively elected by original presentation for prosecution on the merits. Accordingly, claims 31-45 are withdrawn from consideration as being directed to a non-elected invention. See 37 C.F.R. 1.142(b) and M.P.E.P. 821.03.

Claims 15-30 are under consideration as they read on the elected invention and species of anti-C5 antibodies.

4. Applicant's arguments, filed 09/21/2009, have been fully considered but rendered moot in view of the New Grounds of Rejection set forth herein.

5. Priority.

The effective filing date of the instant claims 15-30 is deemed to be the filing date of instant priority application USSN 11/127,438, i.e. 05/11/2005.

The previous priority applications do not support sufficient written description for the instant claims, including limitations such as "aerosol composition … suitable for treating a pulmonary condition in a subject", "biopharmaceutical package … " and "pulmonary drug delivery kit …" as well as various limitations of the dependent claims (e.g., "jet air / ultrasonic / shockwave nebulizer".

Applicant should compare the priority applications with the instant claims for differences in the description of the "compositions / kits / packages", "antibody specificities" and "pulmonary conditions" described.

For example, USSNs 60/469,189 and 60/408,571 are limited to describing the treatment of asthma and not other "pulmonary conditions" in a subject, as currently claimed.
USSN 10/655,861 is limited to treating chronic obstructive pulmonary diseases such as asthma but not generically to "pulmonary conditions", as currently claimed.

Also, "kits . packages" do not appear to be described prior to the instant application.

If applicant desires priority prior to 05/11/2005, applicant is invited to point out and provide documentary support for the priority of the instant claims.

Applicant is reminded that such priority for the instant limitations requires written description and enablement under 35 U.S.C. § 112, first paragraph.

Obviousness is not the standard for the addition new limitations to the disclosure as filed. It is noted that entitlement to a filing date does not extend to subject matter which is not disclosed, but would be obvious over what is expressly disclosed. Lockwood v. American Airlines Inc., 41 USPQ2d 1961 (Fed. Cir. 1977).

A claim as a whole has only one effective filing date.
See Studiengelsellschaft Kahle m.b.H. v. Shell Oil Co. 42 USPQ2d 1674, 1677 (Fed. Cir 1997).

6. The title of the invention is not descriptive. A new title is required that is clearly indicative of the invention to which the claims are directed. Applicant should restrict the title to the claimed invention.

7. The application is required to be reviewed and all spelling, TRADEMARKS, and like errors corrected. Appropriate corrections are required.

Trademarks should be capitalized or accompanied by the ® or ™ symbol wherever they appear and be accompanied by the generic terminology. Although the use of trademarks is permissible in patent applications, the proprietary nature of the trademarks should be respected and every effort made to prevent their use in any manner, which might adversely affect their validity as trademarks.

8. The following is a quotation of the appropriate paragraphs of 35 U.S.C. § 102 that form the basis for the rejections under this section made in this Office Action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

(e) the invention was described in (1) an application for patent, published under section 122(b), by another filed in the United States before the invention by the applicant for patent or (2) a patent granted on an application for patent by another filed in the United States before the invention by the applicant for patent, except that an international application filed under the treaty defined in section 351(a) shall have the effects for purposes of this subsection of an application filed in the United States only if the international application designated the United States and was published under Article 21(2) of such treaty in the English language.

9. The following is a quotation of 35 U.S.C. § 103(a) which forms the basis for all obviousness rejections set forth in this Office Action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negatived by the manner in which the invention was made.

10. Claims 15-17 are rejected under 35 U.S.C. § 102(b) as being anticipated by Wang et al. (WO 2004/022096) (1449; BB) (see entire document).

Wang et al. teach formulations, including aerosol (e.g., pages 20-21, overlapping paragraph and Summary of the Invention) comprising anti-C5 antibodies (e.g., see pages 14-17) that are useful for the treatment of asthma, including combination therapy (e.g., see Summary of the Invention and Detailed Description) (see entire document, including claims).

A species will anticipate a claim to a genus. See MPEP 2131.02.

11. Claims 15-18, 22 and 30 are rejected under 35 U.S.C. § 102(b)(e) as being anticipated by Krause et al. (US 2004/0014782) (see entire document).

Krause et al. teach the use of C5a antagonists, including anti-C5 antibodies (e.g., see paragraphs [0207] and [0277]) in the treat respiratory diseases lung disorders, including ARDS and asthma (e.g., see paragraphs [0039], [0204] [0227, [0274]), including inhaled compositions, nebulizers or other devices that may be provided in a kit for the treatment of asthma (e.g., see paragraphs [0273]—[0275], [0279]-[0281], [0288]-[[0303]) as well as combinations for the treatment of lung disorders (e.g., see paragraphs [0200], [0225]-[0235]) and dosages consistent with the broad range recited in claim 31 (e.g., see paragraphs [0263]-[0300]) (see entire document).

Given that there may be ambiguity over the effective priority date of the instant claims, this rejection is made under 35 U.S.C. 102(b)(e), as it would apply to the priority of the instant claims if applicant can provide appropriate written support and enablement for the instant or possibly amended claims. See Section 5 above concerning priority of the instant claims.

12. Claims 15-30 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Krause et al. (US 2004/0014782) in view of West et al. (U.S. Patent No. 7,071,299), Babington (U.S. Patent No. 4,228,795) (892; of record) and Lobb et al. (U.S. Patent No. 5,871,734) (1449; #AA).

Krause et al. teach the use of C5a antagonists, including anti-C5 antibodies (e.g., see paragraphs [0207] and [0277]) in the treat respiratory diseases lung disorders, including ARDS and asthma (e.g., see paragraphs [0039], [0204] [0227, [0274]), including compositions and pharmaceutical kits for the treatment of asthma (e.g., see paragraphs [0273]—[0275], [0279]-[0281], [0288]-[[0303]) and instructions (e.g., see claim 29, paragraphs 263, [0273], [0278], [0297]) as well as combinations for the treatment of lung disorders (e.g., see paragraphs [0200], [0225]-[0235]) and dosages consistent with the broad range recited in claim 31 (e.g., see paragraphs [0263]-[0300]) (see entire document).

Krause et al. differs from the claimed compositions / packages and kits by not describing the nature of the well known inhalers at the time the invention was made.

West et al. teach compositions comprising complement inhibitors of complement activation for therapy including administration via inhalation, mucosal membrane, pulmonary routes, intranasal delivery with aid of dry-powder dispensers, liquid aerosol generators or nebulizers as well as the provision of such compositions of complement inhibitors in the kit form, with can comprise written information or indication and usage of the pharmaceutical composition (e.g., see Therapeutic Uses of Complement C1s Inhibitors Peptides and Polypeptides on columns 31-35) (see entire document).

Babington teaches nebulizers which can be used to aerosolize medication for nasal inhalation (e.g., see Figure 4 and columns 6-8), which is suitable for use with viscous or sticky substances (e.g., see column 8, lines 34-37) and can use a jet of air to disperse the medicament (e.g., see column 4, lines 35-39 and claims 18, 19) or ultrasonic nebulizers in the art (e.g., see column 1, lines 23-34 and claim 19) (see entire document).

In addition to the teachings above Lobb et al. teach that aqueous antibody solutions can be delivered to airways using a nebulizer (e.g., see column 6, lines 36-41 and column 12, lines 37-52) as well as the use of antibodies for the treatment of asthma (see entire document, including Summary of the Invention). Note, too, that Lobb et al. also teach the well known applicability of combination therapy, including combination having a therapeutic effect on airway responsiveness (e.g., see column 8, paragraphs 4-5).

It would have been prima facie obvious to one of ordinary skill in the art at the time the invention was made to employ the nebulizers taught by West et al. and Babington to administer the anti-C5a antibodies via the aerosol / nebulizer means taught by Krause et al. in the treatment of certain respiratory / lung disorders / diseases such as asthma One would have been motivated with a reasonable expectation of success to administer the antibodies directly to the respiratory mucosa, which is often the first line of encounter of an immune system with pathogenic organisms and by the teachings of the prior art as a known and effective means to target the respiratory system in the treatment of certain disorders/diseases.

With respect to the limitation of comprising "instructions for the use of the composition", the following is noted.
Note, however, that C5 antagonists as well as anti-C5 antibodies are taught herein for the treatment of pulmonary or lung disorders / diseases and that Krause et al. and West et al. both teach the applicability of "instructions".

The printed matter on the label or package insert does not lend patentable weight as a limitation of the claimed invention because the recited intended use does not physically or chemically change the nature of the agent within the container and the artisan can still use the agent for other purposes. The intended use of a product, in this case an anti-C5 antibody, does not materially change the product as the product can still be used for other purposes, such as assaying C5 binding or immunopurification of C5, which are routinely practiced in the art. The recited "limitations" only further limit the intended use method printed on the label or package insert and do not change the physical and/or chemical properties of the components of the kit.

Also, see In re Haller, 73 USPQ 403 (CCPA 1947), In re Venezia, 189 USPQ 149 (CCPA 1976) In re Ngai, 70 USPQ2d 1862 (Fed. Cir. 2004), In re Gulack, 217 USPQ 401, 404 (Fed. Cir. 1983) and MPEP 2112.01
Where the only difference between a prior art product and a claimed product is printed matter that is not functionally related to the product, the content of the printed matter will not distinguish the claimed product from the prior art. In re Ngai, 70 USPQ2d 1862, 1864 (Fed. Cir. 2004).

Since providing C5 antagonists as well as anti-C5 antibodies to ameliorate various pulmonary conditions / diseases such as asthma would have been predictable at the time of the invention, there would have been reasonable expectation of successful development of providing said C5 antagonists alone or in combination with other agents to treat said conditions/diseases in composition or kit form for convenience and economy. The prior art had recognized the advantages of C5 antagonists such as anti-C5 antibodies in the treatment of pulmonary diseases/ disorders and pharmaceutical kits as a standard and convenient means of providing pharmaceutical agents to the ordinary artisan.

"The test of obviousness is not express suggestion of the claimed invention in any or all of the references but rather what the references taken collectively would suggest to those of ordinary skill in the art presumed to be familiar with them." See In re Rosselet, 146 USPQ 183, 186 (CCPA 1965).

"There is no requirement (under 35 USC 103(a)) that the prior art contain an express suggestion to combine known elements to achieve the claimed invention. Rather, the suggestion to combine may come from the prior art, as filtered through the knowledge of one skilled in the art." Motorola, Inc. v. Interdigital Tech. Corp., 43 USPQ2d 1481, 1489 (Fed. Cir. 1997).

An obviousness determination is not the result of a rigid formula disassociated from the consideration of the facts of a case. Indeed, the common sense of those skilled in the art demonstrates why some combinations would have been obvious where others would not. See KSR Int'l Co. v. Teleflex Inc., 82 USPQ2d 1385 (U.S. 2007) ("The combination of familiar elements according to known methods is likely to be obvious when it does no more than yield predictable results.").

Given that the prior art goal was to inhibit complement activation in order to treat pulmonary diseases/conditions,
incorporating known inhibitors such as anti-C5 antibodies into pharmaceutical compositions . kits would have been routine to the ordinary artisan at the time the invention was made and therefore obvious in designing therapeutic products regimens to treat pulmonary diseases / conditions.

From the teachings of the references, a person of ordinary skill in the art would have a reasonable expectation of success at the time the invention was made. Therefore, the invention as a whole was prima facie obvious to one of ordinary skill in the art at the time the invention was made, as evidenced by the references, especially in the absence of evidence to the contrary.

13. No claim is allowed.

14. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Phillip Gambel whose telephone number is (571) 272-0844. The examiner can normally be reached Monday through Thursday from 7:30 am to 6:00 pm. A message may be left on the examiner's voice mail service. If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Ram Shukla can be reached on (571) 272-0735.

The fax number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see http://pair-direct.uspto.gov. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free).

/Phillip Gambel/
Primary Examiner
Technology Center 1600
Art Unit 1644
August 16, 2010

Notice of References Cited	Application/Control No. 11/127,438	Applicant(s)/Patent Under Reexamination WANG, YI	
	Examiner Phillip Gambel	Art Unit 1644	Page 1 of 1

U.S. PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Name	Classification
*	A	US-2004/0014782	01-2004	Krause, James E.	514/313
*	B	US-7,071,299	07-2006	West et al.	530/324
	C	US-			
	D	US-			
	E	US-			
	F	US-			
	G	US-			
	H	US-			
	I	US-			
	J	US-			
	K	US-			
	L	US-			
	M	US-			

FOREIGN PATENT DOCUMENTS

*		Document Number Country Code-Number-Kind Code	Date MM-YYYY	Country	Name	Classification
	N					
	O					
	P					
	Q					
	R					
	S					
	T					

NON-PATENT DOCUMENTS

*		Include as applicable: Author, Title Date, Publisher, Edition or Volume, Pertinent Pages)
	U	
	V	
	W	
	X	

*A copy of this reference is not being furnished with this Office action. (See MPEP § 707.05(a).)
Dates in MM-YYYY format are publication dates. Classifications may be US or foreign.

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File   1:ERIC 1965-2010/Jul
       (c) format only 2010 Dialog

      Set  Items  Description
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Cost is in DialUnits
? b 410
       12aug10 13:26:56 User208760 Session D3205.1
            $0.61    0.160 DialUnits File1
     $0.61  Estimated cost File1
     $0.02  TELNET
     $0.63  Estimated cost this search
     $0.63  Estimated total session cost   0.160 DialUnits

File 410:The Chronolog 1991-2010/ Mar
       (c) 2010 Dialog. All rights reserved.

      Set  Items  Description
      ---  -----  -----------
? set hi ;set hi
HILIGHT set on as ''
HILIGHT set on as ''
? begin 5,73,155,399
       12aug10 13:27:11 User208760 Session D3205.2
            $0.00    0.117 DialUnits File410
     $0.00  Estimated cost File410
     $0.08  TELNET
     $0.08  Estimated cost this search
     $0.71  Estimated total session cost   0.277 DialUnits

SYSTEM:OS  - DIALOG OneSearch
  File   5:Biosis Previews(R) 1926-2010/Aug W2
         (c) 2010 The Thomson Corporation
  File  73:EMBASE 1974-2010/Aug 12
         (c) 2010 Elsevier B.V.
  File 155:MEDLINE(R) 1950-2010/Aug 10
         (c) format only 2010 Dialog
  File 399:CA SEARCH(R) 1967-2010/UD=15307
         (c) 2010 American Chemical Society
*File 399: Use is subject to the terms of your user/customer agreement.
IPCR/8 classification codes now searchable as IC=. See HELP NEWSIPCR.

      Set  Items  Description
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? s (anti(w)c5 or c5)(10n)(antibod?) and (asthma? or bronichitis or emphysema)
         2406597  ANTI
           39959  C5
             479  ANTI(W)C5
           39959  C5
         2739366  ANTIBOD?
            1939  (ANTI(W)C5 OR C5)(10N)ANTIBOD?
          394790  ASTHMA?
               0  BRONICHITIS
           63994  EMPHYSEMA
      S1      19  (ANTI(W)C5 OR C5)(10N)(ANTIBOD?) AND (ASTHMA? OR
                  BRONICHITIS OR EMPHYSEMA)
? rd s1
      S2      19  RD S1  (unique items)
? t s2/3/all
```


Samsung Bioepis Exhibit No. 1002
Page 8555

2/3/1 (Item 1 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2010 The Thomson Corporation. All rts. reserv.

0019486525 BIOSIS NO.: 200700146266
Effect of blocking intrapulmonary activation of C5 on the development of airway hyperresponsiveness (AHR) and airway inflammation
AUTHOR: Peng Tao (Reprint); Su Xiao; Kumor Karen; Liu-Chen Susan; Zhou Helen; Miano Dino; Shen Yamin; Wang Yi
AUTHOR ADDRESS: Alex Pharmaceut Inc, Cheshire, CT 06410 USA**USA
JOURNAL: Molecular Immunology 44 (1-3, Sp. Iss. SI): p227 JAN 2007 2007
CONFERENCE/MEETING: 21st International Complement Workshop Beijing, PEOPLES R CHINA October 22 -26, 2006; 20061022
ISSN: 0161-5890
DOCUMENT TYPE: Meeting; Meeting Abstract
RECORD TYPE: Citation
LANGUAGE: English

2/3/2 (Item 1 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2010 Elsevier B.V. All rts. reserv.

0082532345 EMBASE/Medline No: 2008337552
The spectrum of complement alternative pathway-mediated diseases
Holers V.M.
Department of Medicine and Immunology, University of Colorado Denver School of Medicine, Aurora, CO, United States; Department of Medicine and Immunology, Division of Rheumatology, University of Colorado Denver School of Medicine, P.O. Box 6511, Aurora, CO 80045, United States
AUTHOR EMAIL: michael.holers@uchsc.edu
CORRESP. AUTHOR/AFFIL: Holers V. M.: Department of Medicine and Immunology, Division of Rheumatology, University of Colorado Denver School of Medicine, P.O. Box 6511, Aurora, CO 80045, United States
CORRESP. AUTHOR EMAIL: michael.holers@uchsc.edu

Immunological Reviews (Immunol. Rev.) (United Kingdom) June 1, 2008, 223/1 (300-316)
CODEN: IMRED ISSN: 0105-2896 eISSN: 1600-065X
DOI: 10.1111/j.1600-065X.2008.00641.x
DOCUMENT TYPE: Journal; Review RECORD TYPE: Abstract
LANGUAGE: English SUMMARY LANGUAGE: English
NUMBER OF REFERENCES: 136

2/3/3 (Item 2 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2010 Elsevier B.V. All rts. reserv.

0082137801 EMBASE/Medline No: 2007551026
Complement-targeted therapeutics
Ricklin D.; Lambris J.D.
Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, 422 Curie Blvd., Philadelphia, PA 19104, United States
AUTHOR EMAIL: lambris@mail.med.upenn.edu
CORRESP. AUTHOR/AFFIL: Lambris J.D.: Department of Pathology and Laboratory Medicine, School of Medicine, University of Pennsylvania, 422 Curie Blvd., Philadelphia, PA 19104, United States
CORRESP. AUTHOR EMAIL: lambris@mail.med.upenn.edu

Samsung Bioepis Exhibit No. 1002
Page 8556

Nature Biotechnology (Nat. Biotechnol.) (United States) November 1, 2007, 25/11 (1265-1275)
CODEN: NABIF ISSN: 1087-0156 eISSN: 1546-1696
PUBLISHER ITEM IDENTIFIER: NBT1342
DOI: 10.1038/nbt1342
DOCUMENT TYPE: Journal; Review RECORD TYPE: Abstract
LANGUAGE: English SUMMARY LANGUAGE: English
NUMBER OF REFERENCES: 103

2/3/4 (Item 3 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2010 Elsevier B.V. All rts. reserv.

0082070115 EMBASE/Medline No: 2007504675
Function, structure and therapeutic potential of complement C5a receptors
Monk P.N.; Scola A.-M.; Madala P.; Fairlie D.P.
Academic Neurology Unit, School of Medicine and Biomedical Science, University of Sheffield, Sheffield, United Kingdom; Academic Neurology Unit, School of Medicine and Biomedical Science, University of Sheffield, Beech Hill Road, Sheffield S10 2RX, United Kingdom
AUTHOR EMAIL: p.monk@shef.ac.uk
CORRESP. AUTHOR/AFFIL: Monk P.N.: Academic Neurology Unit, School of Medicine and Biomedical Science, University of Sheffield, Beech Hill Road, Sheffield S10 2RX, United Kingdom
CORRESP. AUTHOR EMAIL: p.monk@shef.ac.uk

British Journal of Pharmacology (Br. J. Pharmacol.) (United Kingdom) October 1, 2007, 152/4 (429-448)
CODEN: BJPCB ISSN: 0007-1188 eISSN: 1476-5381
PUBLISHER ITEM IDENTIFIER: 0707332
DOI: 10.1038/sj.bjp.0707332
DOCUMENT TYPE: Journal; Review RECORD TYPE: Abstract
LANGUAGE: English SUMMARY LANGUAGE: English
NUMBER OF REFERENCES: 259

2/3/5 (Item 4 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2010 Elsevier B.V. All rts. reserv.

0081733479 EMBASE/Medline No: 2007167372
A study for an allergic inflammation model using human lungs and its pharmacological application
Hama H.; Ono N.; Abe M.
Pharmaco-Informatics Research Unit, Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan
AUTHOR EMAIL: ononob@fukuoka-u.ac.jp
CORRESP. AUTHOR/AFFIL: Hama H.: Pharmaco-Informatics Research Unit, Faculty of Pharmaceutical Sciences, Fukuoka University, Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan

Yakugaku Zasshi (Yakugaku Zasshi) (Japan) April 1, 2007, 127/4 (721-727)
CODEN: YKKZA ISSN: 0031-6903 eISSN: 1347-5231
DOI: 10.1248/yakushi.127.721
URL: http://yakushi.pharm.or.jp/FULL TEXT/127 4/pdf/721.pdf
DOCUMENT TYPE: Journal; Review RECORD TYPE: Abstract
LANGUAGE: Japanese SUMMARY LANGUAGE: English
NUMBER OF REFERENCES: 19

Samsung Bioepis Exhibit No. 1002
Page 8557

2/3/6 (Item 5 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2010 Elsevier B.V. All rts. reserv.

0081633412 EMBASE/Medline No: 2007066844
Eculizumab: 5G1.1, h5G1.1, long-acting ***anti*** - ***C5*** monoclonal antibody 5G1-1, long-acting anti-C5 monoclonal
antibody 5G1.1

Drugs in R and D (Drugs R D) (New Zealand) March 16, 2007, 8/1 (61-68)
CODEN: DRDDF ISSN: 1174-5886
DOI: 10.2165/00126839-200708010-00006
URL: http://drugsrd.adisonline.com/pt/re/rdd/pdfhandler.00126839-200708010-00006.pdf;jsessionid=F2Lp8pZfXmXhPxvXBJfnLVHwnsnmrbLncVlh8mGpQW82WCXkYhD3!1416047858!-949856144!8091!-1
DOCUMENT TYPE: Journal; Article RECORD TYPE: Abstract
LANGUAGE: English SUMMARY LANGUAGE: English
NUMBER OF REFERENCES: 23

2/3/7 (Item 6 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2010 Elsevier B.V. All rts. reserv.

0080620746 EMBASE/Medline No: 2005265038
Role of C5 in the development of airway inflammation, airway hyperresponsiveness, and ongoing airway response
Peng T.; Hao L.; Madri J.A.; Su X.; Elias J.A.; Stahl G.L.; Squinto S.; Wang Y.
Alexion Pharmaceuticals Inc., Cheshire, CT, United States
AUTHOR EMAIL: wangy@alxn.com
CORRESP. AUTHOR/AFFIL: Wang Y.: Alexion Pharmaceuticals Inc., 352 Knotter Drive, Cheshire, CT 06410, United States
CORRESP. AUTHOR EMAIL: wangy@alxn.com

Journal of Clinical Investigation (J. Clin. Invest.) (United States) June 1, 2005, 115/6 (1590-1600)
CODEN: JCINA ISSN: 0021-9738
DOI: 10.1172/JCI22906
DOCUMENT TYPE: Journal; Article RECORD TYPE: Abstract
LANGUAGE: English SUMMARY LANGUAGE: English
NUMBER OF REFERENCES: 30

2/3/8 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

18538271 PMID: 18424754 Record Identifier: NIHMS93302; PMC2645414
Generation of anaphylatoxins by human beta-tryptase from C3, C4, and C5.
Fukuoka Yoshihiro; Xia Han-Zhang; Sanchez-Munoz Laura B; Dellinger Anthony L; Escribano Luis; Schwartz Lawrence B
Division of Rheumatology, Allergy and Immunology, Department of Internal Medicine, Virginia Commonwealth University, Richmond, VA 23298, USA.
Journal of immunology (Baltimore, Md. - 1950) (United States) May 1 2008, 180 (9) p6307-16, ISSN 0022-1767--Print 0022-1767--Linking Journal Code: 2985117R
Contract/Grant No.: R01 AI 27517; AI; NIAID NIH HHS United States; R01

Samsung Bioepis Exhibit No. 1002
Page 8558

AI020487-22; AI; NIAID NIH HHS United States; R01 AI027517-18; AI; NIAID NIH HHS United States; U19 AI077435-010001; AI; NIAID NIH HHS United States Publishing Model Print; Cites J Immunol. 2003 Feb 15;170(4):1625-9 PMID 12574323; Cites Eur J Immunol. 2003 Apr;33(4):899-906 PMID 12672055; Cites J Immunol. 2003 Jun 1;170(11):5667-73 PMID 12759448; Cites Leukemia. 2003 Jul;17(7):1428-30 PMID 12835741; Cites Blood. 2003 Oct 1;102(7):2547-54 PMID 12855579; Cites Am J Respir Crit Care Med. 2003 Dec 1;168(11):1333-41 PMID 14500265; Cites Am J Respir Cell Mol Biol. 2004 Aug;31(2):216-9 PMID 15039137; Cites J Immunol. 2004 Jul 1;173(1):367-75 PMID 15210795; Cites J Clin Immunol. 1994 May;14(3):190-204 PMID 7929694; Cites J Immunol. 1970 Mar;104(3):535-43 PMID 4985169; Cites Annu Rev Immunol. 1994;12:775-808 PMID 8011297; Cites Lab Invest. 1993 Nov;69(5):552-61 PMID 8246447; Cites J Clin Invest. 1996 Feb 15;97(4):988-95 PMID 8613553; Cites Clin Exp Allergy. 1997 Apr;27(4):396-405 PMID 9146932; Cites J Clin Invest. 1997 Jun 1;99(11):2691-700 PMID 9169499; Cites J Allergy Clin Immunol. 1997 Aug;100(2):253-60 PMID 9275149; Cites J Immunol. 1997 Oct 1;159(7):3540-8 PMID 9317153; Cites Immunopharmacology. 1997 Dec;38(1-2):3-15 PMID 9476110; Cites Nature. 1998 Mar 19;392(6673):306-11 PMID 9521329; Cites J Allergy Clin Immunol. 2004 Jul;114(1):174-82 PMID 15241362; Cites Biochemistry. 2004 Aug 24;43(33):10757-64 PMID 15311937; Cites J Immunol Methods. 1992 Mar 4;147(2):241-50 PMID 1548406; Cites J Asthma. 2004 Sep;41(6):631-8 PMID 15584312; Cites Biochemistry. 2005 Apr 26;44(16):6239-49 PMID 15835912; Cites J Allergy Clin Immunol. 2005 Jun;115(6):1148-54 PMID 15940127; Cites J Allergy Clin Immunol. 2005 Jun;115(6):1162-8 PMID 15940129; Cites Blood. 2006 Jan 15;107(2):610-8 PMID 16174756; Cites J Immunol. 2006 Mar 1;176(5):3165-72 PMID 16493076; Cites J Invest Dermatol. 2006 Jun;126(6):1403-9 PMID 16543894; Cites Nat Med. 2006 Jun;12(6):682-7 PMID 16715088; Cites J Allergy Clin Immunol. 2006 Jun;117(6):1277-84 PMID 16750987; Cites J Allergy Clin Immunol. 2006 Jun;117(6):1411-4 PMID 16751005; Cites J Immunol. 2006 Jul 1;177(1):694-701 PMID 16785568; Cites Arch Biochem Biophys. 1990 Jan;276(1):26-31 PMID 1688695; Cites Immunol Rev. 2007 Jun;217:141-54 PMID 17498057; Cites J Immunol. 1990 Mar 15;144(6):2304-11 PMID 2179409; Cites Exp Cell Res. 1990 May;188(1):42-9 PMID 2328776; Cites Am Rev Respir Dis. 1990 Jul;142(1):126-32 PMID 2368958; Cites Am Rev Respir Dis. 1988 May;137(5):1002-8 PMID 2461667; Cites J Immunol. 1988 Apr 15;140(8):2708-13 PMID 3356902; Cites Biochem Soc Symp. 1986;51:69-81 PMID 3545212; Cites J Immunol. 1979 Oct;123(4):1445-50 PMID 479592; Cites J Clin Invest. 1979 Dec;64(6):1537-43 PMID 500822; Cites J Exp Med. 1983 Jun 1;157(6):2029-48 PMID 6222137; Cites J Immunol. 1983 Apr;130(4):1891-5 PMID 6339618; Cites J Invest Dermatol. 1980 Apr;74(4):210-5 PMID 6445388; Cites J Exp Med. 1981 Jun 1;153(6):1391-404 PMID 6910482; Cites J Immunol. 1981 Apr;126(4):1290-4 PMID 7009736; Cites Biochemistry. 1981 Jul 21;20(15):4458-67 PMID 7284336; Cites J Invest Dermatol. 1994 May;102(5):803-6 PMID 7513741; Cites Lab Invest. 1995 Feb;72(2):215-22 PMID 7531794; Cites Invasion Metastasis. 1994-1995;14(1-6):395-408 PMID 7544776; Cites Am J Respir Crit Care Med. 1995 Mar;151(3 Pt 1):624-9 PMID 7881647; Cites Exp Dermatol. 1999 Feb;8(1):1-16 PMID 10206716; Cites Genes Dev. 1999 Jun 1;13(11):1382-97 PMID 10364156; Cites J Invest Dermatol. 2000 Jan;114(1):51-5 PMID 10620115; Cites Int J Cancer. 2000 Jan 15;85(2):171-5 PMID 10629073; Cites J Pathol. 2000 Jan;190(1):100-6 PMID 10640999; Cites Am J Respir Crit Care Med. 2000 Mar;161(3 Pt 1):694-9 PMID 10712309; Cites Dermatol Surg. 2000 Mar;26(3):200-3; discussion 203-4 PMID 10759793; Cites Cancer. 2000 Jun 15;88(12):2686-92 PMID 10870050; Cites Eur Respir J. 2000 Jun;15(6):1087-93 PMID 10885428; Cites Exp Dermatol. 2000 Aug;9(4):258-65 PMID 10949547; Cites Nat Immunol. 2000 Sep;1(3):221-6 PMID 10973279; Cites Nature. 2000 Aug 31;406(6799):998-1001 PMID 10984054; Cites J Immunol. 2001 Feb 1;166(3):2025-32 PMID 11160252; Cites Blood. 2001 Apr 1;97(7):2045-52 PMID 11264170; Cites Am J Respir Crit Care Med. 2001 Nov 15;164(10 Pt 1):1841-3 PMID 11734433; Cites N Engl J Med. 2002 May

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Page 8559

30;346(22):1699-705 PMID 12037149; Cites Thorax. 2002 Aug;57(8):677-82 PMID 12149526; Cites J Magn Reson Imaging. 2002 Oct;16(4):430-50 PMID 12353258; Cites Biochem J. 2003 Feb 1;369(Pt 3):603-10 PMID 12387726; Cites Am J Pathol. 2002 Nov;161(5):1849-59 PMID 12414531
Document type: Journal Article; Research Support, N.I.H., Extramural; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Other Citation Owner: NLM; NLM
Record type: MEDLINE; Completed

2/3/9 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

14155560 PMID: 11159035
Complement-dependent immune complex-induced bronchial inflammation and hyperreactivity.
Lukacs N W; Glovsky M M; Ward P A
Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0602, USA. nlukacs@umich.edu
American journal of physiology. Lung cellular and molecular physiology (United States) Mar 2001, 280 (3) pL512-8, ISSN 1040-0605--Print 1040-0605--Linking Journal Code: 100901229
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

2/3/10 (Item 3 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

06742935 PMID: 6459046
Immune complex injury of the lung. Symposium held at the 74th annual meeting of the American Thoracic Society, Las Vegas, Nevada, May 1979.
Daniele R P; Henson P M; Fantone J C; Ward P A; Dreisin R B
American review of respiratory disease (UNITED STATES) Dec 1981, 124 (6) p738-55, ISSN 0003-0805--Print 0003-0805--Linking Journal Code: 0370523
Contract/Grant No.: AI-09651; AI; NIAID NIH HHS United States; HL-21565; HL; NHLBI NIH HHS United States; HL-23877; HL; NHLBI NIH HHS United States; +
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't; Research Support, U.S. Gov't, P.H.S.; Review
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

2/3/11 (Item 1 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
(c) 2010 American Chemical Society. All rts. reserv.

152236893 CA: 152(11)236893b PATENT
Anti-human complement C5 antibodies and fragments for treatment of age-related macular degeneration and other C5-associated diseases

Samsung Bioepis Exhibit No. 1002
Page 8560

INVENTOR(AUTHOR): Diefenbach-Streiber, Beate; Eberth, Adina; Guild, Braydon Charles; Kim, Yong-In; Roguska, Michael; Splawski, Igor
LOCATION: Switz.
ASSIGNEE: Novartis AG
PATENT: PCT International ; WO 201015608 A1 DATE: 20100211
APPLICATION: WO 2009EP60052 (20090803) *US 2008PV86355 (20080805)
PAGES: 218pp. CODEN: PIXXD2 LANGUAGE: English
PATENT CLASSIFICATIONS:

IPCR/8 + Level	Value	Position	Status	Version	Action	Source	Office:
A61K-0039/395	A	I	F	B	20060101	H	EP
C07K-0016/18	A	I	L	B	20060101	H	EP

DESIGNATED COUNTRIES: AE; AG; AL; AM; AO; AT; AU; AZ; BA; BB; BG; BH; BR; BW; BY; BZ; CA; CH; CL; CN; CO; CR; CU; CZ; DE; DK; DM; DO; DZ; EC; EE; EG; ES; FI; GB; GD; GE; GH; GM; GT; HN; HR; HU; ID; IL; IN; IS; JP; KE; KG; KM; KN; KP; KR; KZ; LA; LC; LK; LR; LS; LT; LU; LY; MA; MD; ME; MG; MK; MN; MW; MX; MY; MZ; NA; NG; NI; NO; NZ; OM; PE; PG; PH; PL; PT; RO; RS; RU; SC; SD; SE; SG; SK; SL; SM; ST; SV; SY; TJ; TM DESIGNATED REGIONAL: AT; BE; BG; CH ; CY; CZ; DE; DK; EE; ES; FI; FR; GB; GR; HR; HU; IE; IS; IT; LT; LU; LV; MC; MK; MT; NL; NO; PL; PT; RO; SE; SI; SK; SM; TR; BF; BJ; CF; CG; CI; CM; GA; GN; GQ; GW; ML; MR; NE; SN; TD; TG; BW; GH; GM; KE; LS; MW; MZ; NA; SD; SL; SZ; TZ; UG; ZM; ZW; AM; AZ; BY; KG; KZ; MD; RU; TJ; TM

2/3/12 (Item 2 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
(c) 2010 American Chemical Society. All rts. reserv.

151286777 CA: 151(13)286777s JOURNAL
The effect of C5 inhibition by eculizumab on allergen-induced asthmatic responses in patients
AUTHOR(S): Gauvreau, Gail; Boulet, Louis-Philippe; Severino, Beth; Watson, Rick; Peng, Tao; Obminski, George; Prince, Philippe; Deschesnes, Francine; Strinich, Tara; Killian, K. J.; Cote, Joanne; O'Byrne, Paul; Wang, Yi
LOCATION: Department of Medicine, Faculty of Health Sciences, McMaster University, Hamilton, ON, Can.,
JOURNAL: Mol. Immunol. (Molecular Immunology) DATE: 2009 VOLUME: 46
NUMBER: 14 PAGES: 2837-2838 CODEN: MOIMD5 ISSN: 0161-5890
PUBLISHER ITEM IDENTIFIER: 0161-5890(09)00480-5 LANGUAGE: English
PUBLISHER: Elsevier Ltd.

2/3/13 (Item 3 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
(c) 2010 American Chemical Society. All rts. reserv.

150054225 CA: 150(4)54225m PATENT
Antibodies specific for complement C3b, their sequences and use in prevention and treatment of complement-associated disorders, such as inflammatory or autoimmune diseases
INVENTOR(AUTHOR): Van Lookeren Campagne, Menno
LOCATION: USA
ASSIGNEE: Genentech, Inc.
PATENT: PCT International ; WO 2008154251 A2 DATE: 20081218
APPLICATION: WO 2008US65771 (20080604) *US 2007PV933721 (20070607) *US 2008PV55068 (20080521)
PAGES: 86pp. CODEN: PIXXD2 LANGUAGE: English
PATENT CLASSIFICATIONS:

IPCR/8 + Level	Value	Position	Status	Version	Action	Source	Office:
C07K-0016/18	A	I	F	B	20060101	H	EP
C12N-0015/13	A	I	L	B	20060101	H	EP

Samsung Bioepis Exhibit No. 1002
Page 8561

A61K-0039/395 A I L B 20060101 H EP
A61P-0029/00 A I L B 20060101 H EP
DESIGNATED COUNTRIES: AE; AG; AL; AM; AO; AT; AU; AZ; BA; BB; BG; BH; BR; BW; BY; BZ; CA; CH; CN; CO; CR; CU; CZ; DE; DK; DM; DO; DZ; EC; EE; EG; ES; FI; GB; GD; GE; GH; GM; GT; HN; HR; HU; ID; IL; IN; IS; JP; KE; KG; KM; KN; KP; KR; KZ; LA; LC; LK; LR; LS; LT; LU; LY; MA; MD; ME; MG; MK; MN; MW; MX; MY; MZ; NA; NG; NI; NO; NZ; OM; PG; PH; PL; PT; RO; RS; RU; SC; SD; SE; SG; SK; SL; SM; SV; SY; TJ; TM; TN; TR; TT DESIGNATED REGIONAL: AT; BE; BG; CH; CY; CZ; DE; DK; EE; ES; FI; FR; GB; GR; HR; HU; IE; IS; IT; LT; LU; LV; MC; MT; NL; NO; PL; PT; RO; SE; SI; SK; TR; BF; BJ; CF; CG; CI; CM; GA; GN; GQ; GW; ML; MR; NE; SN; TD; TG; BW; GH; GM; KE; LS; MW; MZ; NA; SD; SL; SZ; TZ; UG; ZM; ZW; AM; AZ; BY; KG; KZ; MD; RU; TJ; TM

2/3/14 (Item 4 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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149183623 CA: 149(8)183623t PATENT
Anti-IL-13 antibody formulations and uses thereof
INVENTOR(AUTHOR): Barry, Anthony B.; Crowley, Thomas J.; Dixon, Daniel A.; Soley, Erin Christine
LOCATION: USA
ASSIGNEE: Wyeth, John, and Brother Ltd.
PATENT: PCT International ; WO 200886395 A2 DATE: 20080717
APPLICATION: WO 2008US50582 (20080109) *US 2007PV879500 (20070109)
PAGES: 106pp. CODEN: PIXXD2 LANGUAGE: English
PATENT CLASSIFICATIONS:
IPCR/8 + Level Value Position Status Version Action Source Office:
A61P-0011/06 A I F B 20060101 H EP
A61P-0037/00 A I L B 20060101 H EP
A61K-0039/395 A I L B 20060101 H EP
C07K-0016/24 A I L B 20060101 H EP
DESIGNATED COUNTRIES: AE; AG; AL; AM; AO; AT; AU; AZ; BA; BB; BG; BH; BR; BW; BY; BZ; CA; CH; CN; CO; CR; CU; CZ; DE; DK; DM; DO; DZ; EC; EE; EG; ES; FI; GB; GD; GE; GH; GM; GT; HN; HR; HU; ID; IL; IN; IS; JP; KE; KG; KM; KN; KP; KR; KZ; LA; LC; LK; LR; LS; LT; LU; LY; MA; MD; ME; MG; MK; MN; MW; MX; MY; MZ; NA; NG; NI; NO; NZ; OM; PG; PH; PL; PT; RO; RS; RU; SC; SD; SE; SG; SK; SL; SM; SV; SY; TJ; TM; TN; TR; TT DESIGNATED REGIONAL: AT; BE; BG; CH; CY; CZ; DE; DK; EE; ES; FI; FR; GB; GR; HR; HU; IE; IS; IT; LT; LU; LV; MC; MT; NL; NO; PL; PT; RO; SE; SI; SK; TR; BF; BJ; CF; CG; CI; CM; GA; GN; GQ; GW; ML; MR; NE; SN; TD; TG; BW; GH; GM; KE; LS; MW; MZ; NA; SD; SL; SZ; TZ; UG; ZM; ZW; AM; AZ; BY; KG; KZ; MD; RU; TJ; TM

2/3/15 (Item 5 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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148119180 CA: 148(6)119180y PATENT
Genetic markers in tachykinin NK1 receptor gene TACR1 that correlate with asthma disorders
INVENTOR(AUTHOR): Halapi, Eva; Hakonarson, Hakon
LOCATION: USA
ASSIGNEE: Decode Genetics Ehf.
PATENT: PCT International ; WO 200806105 A2 DATE: 20080110
APPLICATION: WO 2007US73066 (20070709) *US 2006PV819198 (20060707)
PAGES: 133pp. CODEN: PIXXD2 LANGUAGE: English
PATENT CLASSIFICATIONS:
IPCR/8 + Level Value Position Status Version Action Source Office:
C12Q-0001/68 A I F B 20060101 H EP

Samsung Bioepis Exhibit No. 1002
Page 8562

DESIGNATED COUNTRIES: AE; AG; AL; AM; AT; AU; AZ; BA; BB; BG; BH; BR; BW; BY; BZ; CA; CH; CN; CO; CR; CU; CZ; DE; DK; DM; DO; DZ; EC; EE; EG; ES; FI; GB; GD; GE; GH; GM; GT; HN; HR; HU; ID; IL; IN; IS; JP; KE; KG; KM; KN; KP; KR; KZ; LA; LC; LK; LR; LS; LT; LU; LY; MA; MD; ME; MG; MK; MN; MW; MX; MY; MZ; NA; NG; NI; NO; NZ; OM; PG; PH; PL; PT; RO; RS; RU; SC; SD; SE; SG; SK; SL; SM; SV; SY; TJ; TM; TN; TR; TT; TZ DESIGNATED REGIONAL: AT; BE; BG; CH ; CY; CZ; DE; DK; EE; ES; FI; FR; GB; GR; HU; IE; IS; IT; LT; LU; LV; MC; MT; NL; PL; PT; RO; SE; SI; SK; TR; BF; BJ; CF; CG; CI; CM; GA; GN; GQ; GW; ML; MR; NE; SN; TD; TG; BW; GH; GM; KE; LS; MW; MZ; NA; SD; SL; SZ; TZ; UG; ZM; ZW; AM; AZ; BY; KG; KZ; MD; RU; TJ; TM

2/3/16 (Item 6 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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145333794 CA: 145(17)333794h JOURNAL
A protective role for the fifth complement component (C5) in allergic airway disease
AUTHOR(S): Drouin, Scott M.; Sinha, Meenal; Sfyroera, Georgia; Lambris, John D.; Wetsel, Rick A.
LOCATION: Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas Health Science Center at Houston, Houston, TX, USA
JOURNAL: Am. J. Respir. Crit. Care Med. (American Journal of Respiratory and Critical Care Medicine) DATE: 2006 VOLUME: 173 NUMBER: 8 PAGES: 852-857 CODEN: AJCMED ISSN: 1073-449X LANGUAGE: English PUBLISHER: American Thoracic Society

2/3/17 (Item 7 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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142315237 CA: 142(17)315237n PATENT
Complement C5-binding peptides for inhibiting complement activation and preventing and treating infection and injury
INVENTOR(AUTHOR): Johnson, Richard J.; Maves, Shelley A.
LOCATION: USA
ASSIGNEE: Baxter International Inc.
PATENT: PCT International ; WO 200523866 A2 DATE: 20050317
APPLICATION: WO 2004US29790 (20040910) *US 2003PV501710 (20030910)
PAGES: 113 pp. CODEN: PIXXD2 LANGUAGE: English
PATENT CLASSIFICATIONS:
CLASS: C07K-016/00A
DESIGNATED COUNTRIES: AE; AG; AL; AM; AT; AU; AZ; BA; BB; BG; BR; BW; BY; BZ; CA; CH; CN; CO; CR; CU; CZ; DE; DK; DM; DZ; EC; EE; EG; ES; FI; GB; GD; GE; GH; GM; HR; HU; ID; IL; IN; IS; JP; KE; KG; KP; KR; KZ; LC; LK; LR; LS; LT; LU; LV; MA; MD; MG; MK; MN; MW; MX; MZ; NA; NI; NO; NZ; OM; PG; PH; PL; PT; RO; RU; SC; SD; SE; SG; SK; SL; SY; TJ; TM; TN; TR; TT; TZ; UA; UG; US; UZ; VC; VN; YU; ZA; ZM; ZW DESIGNATED REGIONAL: BW; GH; GM; KE; LS; MW; MZ ; NA; SD; SL; SZ; TZ; UG; ZM; ZW; AM; AZ; BY; KG; KZ; MD; RU; TJ; TM; AT; BE; BG; CH; CY; CZ; DE; DK; EE; ES; FI; FR; GB; GR; HU; IE; IT; LU; MC; NL; PL; PT; RO; SE; SI; SK; TR; BF; BJ; CF; CG; CI; CM; GA; GN; GQ; GW; ML; MR; NE; SN; TD; TG

2/3/18 (Item 8 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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Samsung Bioepis Exhibit No. 1002
Page 8563

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  140252317    CA: 140(16)252317z    PATENT
  Antibodies specific to complement c5 in combination with other
  anti-asthmatic agent for treating or preventing asthma and related airway
  diseases
  INVENTOR(AUTHOR): Wang, Yi
  LOCATION: USA
  ASSIGNEE: Alexion Pharmaceuticals, Inc.
  PATENT: PCT International ; WO 200422096 A1  DATE: 20040318
  APPLICATION: WO 2003US27808 (20030905) *US PV408571 (20020906) *US
PV469189 (20030509)
  PAGES: 58 pp.  CODEN: PIXXD2  LANGUAGE: English
  PATENT CLASSIFICATIONS:
    CLASS:  A61K-039/395A; C07K-016/36B
  DESIGNATED COUNTRIES: AE; AG; AL; AM; AT; AU; AZ; BA; BB; BG; BR; BY; BZ;
CA; CH; CN; CO; CR; CU; CZ; DE; DK; DM; DZ; EC; EE; ES; FI; GB; GD; GE; GH;
GM; HR; HU; ID; IL; IN; IS; JP; KE; KG; KP; KR; KZ; LC; LK; LR; LS; LT; LU;
LV; MA; MD; MG; MK; MN; MW; MX; MZ; NI; NO; NZ; OM; PG; PH; PL; PT; RO; RU;
SC; SD; SE; SG; SK; SL; SY; TJ; TM; TN; TR; TT; TZ; UA; UG; US; UZ; VC; VN;
YU; ZA; ZM; ZW; AM; AZ; BY; KG; KZ; MD; RU  DESIGNATED REGIONAL: GH; GM; KE
; LS; MW; MZ; SD; SL; SZ; TZ; UG; ZM; ZW; AT; BE; BG; CH; CY; CZ; DE; DK;
EE; ES; FI; FR; GB; GR; HU; IE; IT; LU; MC; NL; PT; RO; SE; SI; SK; TR; BF;
BJ; CF; CG; CI; CM; GA; GN; GQ; GW; ML; MR; NE; SN; TD; TG


 2/3/19     (Item 9 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
(c) 2010 American Chemical Society. All rts. reserv.

  136068714    CA: 136(5)68714t    PATENT
  Peptide and polypeptide inhibitors of complement C1s
  INVENTOR(AUTHOR): West, Robert R.; Sheppard, Paul O.; Fox, Brian A.
  LOCATION: USA
  ASSIGNEE: Zymogenetics, Inc.
  PATENT: PCT International ; WO 200198365 A2  DATE: 20011227
  APPLICATION: WO 2001US19405 (20010618) *US PV212998 (20000621)
  PAGES: 99 pp.  CODEN: PIXXD2  LANGUAGE: English
  PATENT CLASSIFICATIONS:
    CLASS:  C07K-700/A
  DESIGNATED COUNTRIES: AE; AG; AL; AM; AT; AU; AZ; BA; BB; BG; BR; BY; BZ;
CA; CH; CN; CO; CR; CU; CZ; DE; DK; DM; DZ; EC; EE; ES; FI; GB; GD; GE; GH;
GM; HR; HU; ID; IL; IN; IS; JP; KE; KG; KP; KR; KZ; LC; LK; LR; LS; LT; LU;
LV; MA; MD; MG; MK; MN; MW; MX; MZ; NO; NZ; PL; PT; RO; RU; SD; SE; SG; SI;
SK; SL; TJ; TM; TR; TT; TZ; UA; UG; UZ; VN; YU; ZA; ZW; AM; AZ; BY; KG; KZ;
MD; RU; TJ; TM  DESIGNATED REGIONAL: GH; GM; KE; LS; MW; MZ; SD; SL; SZ; TZ
; UG; ZW; AT; BE; CH; CY; DE; DK; ES; FI; FR; GB; GR; IE; IT; LU; MC; NL;
PT; SE; TR; BF; BJ; CF; CG; CI; CM; GA; GN; GW; ML; MR; NE; SN; TD; TG
? ds

Set     Items   Description
S1         19   (ANTI(W)C5 OR C5)(10N)(ANTIBOD?) AND (ASTHMA? OR BRONICHIT-
              IS OR EMPHYSEMA)
S2         19   RD S1  (unique items)
? s (c5)(20n)(nebulizer? or aerosol)
>>>Operator "(5C)" in invalid position
? s (nebulizer? or aerosol)(20n)c5)
>>>Unmatched parentheses
? s (nebulizer? or aerosol)(20n)(c5)
>>>Operator "(5C)" in invalid position
? s (nebulizer? or aerosol)(20n)(c5 or anti(W)c5)
           18384  NEBULIZER?
          124040  AEROSOL
```


Samsung Bioepis Exhibit No. 1002
Page 8564

```
          39959  C5
         2406597  ANTI
          39959  C5
            479  ANTI(W)C5
      S3     33  (NEBULIZER? OR AEROSOL)(20N)(C5 OR ANTI(W)C5)
? rd s3
      S4     24  RD S3  (unique items)
? t s4/3/all

 4/3/1     (Item 1 from file: 5)
DIALOG(R)File   5:Biosis Previews(R)
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0020844756   BIOSIS NO.: 200900185090
COUGH REFLEX SENSITIVITY TESTING IN SEASONAL ALLERGIC RHINITIS PATIENTS AND
  HEALTHY VOLUNTEERS
AUTHOR: Pecova R (Reprint); Zucha J; Pec M; Neuschlova M; Hanzel R; Tatar M
AUTHOR ADDRESS: Comenius Univ, Dept Pathol Physiol, Jessenius Fac Med
  Martin, Sklabinska 26 St, Martin 03753, Slovakia**Slovakia
AUTHOR E-MAIL ADDRESS: pecova@jfmed.uniba.sk
JOURNAL: Journal of Physiology and Pharmacology  59 (Suppl. 6, Part 2): p
557-564 DEC 2008 2008
ISSN: 0867-5910
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English


 4/3/2     (Item 2 from file: 5)
DIALOG(R)File   5:Biosis Previews(R)
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0020160269   BIOSIS NO.: 200800207208
Complement C5a anaphylatoxin is an innate determinant of dendritic
  cell-induced Th1 immunity to Mycobacterium bovis BCG infection in mice
AUTHOR: Moulton Rachel A; Mashruwala Mary Anne; Smith Amanda K; Lindsey
  Devin R; Wetsel Rick A; Haviland David L; Hunter Robert L; Jagannath
  Chinnaswamy (Reprint)
AUTHOR ADDRESS: Univ Texas Hlth Ctr, Dept Pathol, Lab Med, 6431 Fannin,
  Houston, TX 77030 USA**USA
AUTHOR E-MAIL ADDRESS: chinnaswamy.jagannath@uth.tmc.edu
JOURNAL: Journal of Leukocyte Biology  82 (4): p956-967 OCT 2007 2007
ITEM IDENTIFIER: doi:10.1189/jlb.0206119
ISSN: 0741-5400
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English


 4/3/3     (Item 3 from file: 5)
DIALOG(R)File   5:Biosis Previews(R)
(c) 2010 The Thomson Corporation. All rts. reserv.

17749188   BIOSIS NO.: 200400119945
Effect of the second-generation antihistamine, fexofenadine, on cough
  reflex sensitivity and pulmonary function.
AUTHOR: Dicpinigaitis Peter V (Reprint); Gayle Yvonne E
AUTHOR ADDRESS: Einstein Division, Montefiore Medical Center, 1825
  Eastchester Road, Bronx, NY, 10461, USA**USA
AUTHOR E-MAIL ADDRESS: pdicpinigaitis@pol.net
JOURNAL: British Journal of Clinical Pharmacology  56 (5): p501-504
```


Samsung Bioepis Exhibit No. 1002
Page 8565

November 2003 2003
MEDIUM: print
ISSN: 0306-5251
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

4/3/4 (Item 4 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
(c) 2010 The Thomson Corporation. All rts. reserv.

16938721 BIOSIS NO.: 200200532232
Dicarboxylic acids in the Arctic aerosols and snowpacks collected during ALERT 2000
AUTHOR: Narukawa M; Kawamura K (Reprint); Li S-M; Bottenheim J W
AUTHOR ADDRESS: Institute of Low Temperature Science, Hokkaido University, NI9 W8 Kita-ku, Sapporo, 060-0819, Japan**Japan
JOURNAL: Atmospheric Environment 36 (15-16): p2491-2499 May-June, 2002 2002
MEDIUM: print
ISSN: 1352-2310
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

4/3/5 (Item 5 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
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16317395 BIOSIS NO.: 200100489234
Characterization of secondary aerosol from the photooxidation of toluene in the presence of NOx and 1-propene
AUTHOR: Jang Myoseon (Reprint); Kamens Richard M
AUTHOR ADDRESS: Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill, Rosenau Hall, Chapel Hill, NC, 27599, USA**USA
JOURNAL: Environmental Science and Technology 35 (18): p3626-3639 September 15, 2001 2001
MEDIUM: print
ISSN: 0013-936X
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: English

4/3/6 (Item 6 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
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08207430 BIOSIS NO.: 198682053817
LUNG DEFENSES AGAINST PSEUDOMONAS-AERUGINOSA IN C-5-DEFICIENT MICE WITH DIFFERENT GENETIC BACKGROUNDS
AUTHOR: CERQUETTI M C (Reprint); SORDELLI D O; BELLANTI J A; HOOKE A M
AUTHOR ADDRESS: GEORGETOWN UNIV SCH MED DENTISTRY, WASHINGTON, DC 20007, USA**USA
JOURNAL: Infection and Immunity 52 (3): p853-857 1986
ISSN: 0019-9567
DOCUMENT TYPE: Article
RECORD TYPE: Abstract

Samsung Bioepis Exhibit No. 1002
Page 8566

LANGUAGE: ENGLISH

4/3/7 (Item 7 from file: 5)
DIALOG(R)File 5:Biosis Previews(R)
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07786516 BIOSIS NO.: 198580095411
EFFECT OF CHEMOTACTINS RELEASED BY STAPHYLOCOCCUS-AUREUS AND PSEUDOMONAS-AERUGINOSA ON THE MURINE RESPIRATORY TRACT
AUTHOR: SORDELLI D O (Reprint); CERQUETTI M C; HOOKE A M; BELLANTI J A
AUTHOR ADDRESS: INTERNATIONAL CENTER FOR INTERDISCIPLINARY STUDIES OF IMMUNOLOGY, GEORGETOWN UNIV SCH OF MED, WASHINGTON, DC 20007, USA**USA
JOURNAL: Infection and Immunity 49 (2): p265-269 1985
ISSN: 0019-9567
DOCUMENT TYPE: Article
RECORD TYPE: Abstract
LANGUAGE: ENGLISH

4/3/8 (Item 1 from file: 73)
DIALOG(R)File 73:EMBASE
(c) 2010 Elsevier B.V. All rts. reserv.

0073164881 EMBASE/Medline No: 1986153915
Lung defenses against Pseudomonas aeruginosa in C5-deficient mice with different genetic backgrounds
Cerquetti M.C.; Sordelli D.O.; Bellanti J.A.; Hooke A.M.
Georgetown University Schools of Medicine and Dentistry, Washington, DC 20007, United States:
CORRESP. AUTHOR/AFFIL: Georgetown University Schools of Medicine and Dentistry, Washington, DC 20007, United States

Infection and Immunity (INFECT. IMMUN.) (United States) August 6, 1986 , 52/3 (853-857)
CODEN: INFIB ISSN: 0019-9567
DOCUMENT TYPE: Journal; Article RECORD TYPE: Abstract
LANGUAGE: English

4/3/9 (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2010 Dialog. All rts. reserv.

33971185 PMID: 20659255
Remote sensing detection of droughts in Amazonian forest canopies.
Anderson Liana O; Malhi Yadvinder; Aragao Luiz E O C; Ladle Richard; Arai Egidio; Barbier Nicolas; Phillips Oliver
Environmental Change Institute, School of Geography and Environment, Oxford University, Oxford, UK. liana.anderson@ouce.ox.ac.uk
New phytologist (England) Aug 2010, 187 (3) p733-50, ISSN 1469-8137 --Electronic 0028-646X--Linking Journal Code: 9882884
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: In Process

4/3/10 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

Samsung Bioepis Exhibit No. 1002
Page 8567

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19013358 PMID: 19218681
Cough reflex sensitivity testing in in seasonal allergic rhinitis patients and healthy volunteers.
Pecova R; Zucha J; Pec M; Neuschlova M; Hanzel P; Tatar M
Department of Pathological Physiology, Comenius University in Bratislava, Jessenius Faculty of Medicine in Martin, Slovakia. pecova@jfmed.uniba.sk
Journal of physiology and pharmacology - an official journal of the Polish Physiological Society (Poland) Dec 2008, 59 Suppl 6 p557-64, ISSN 1899-1505--Electronic 0867-5910--Linking Journal Code: 9114501
Publishing Model Print
Document type: Journal Article; Research Support, Non-U.S. Gov't
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

4/3/11 (Item 1 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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153044265 CA: 153(2)44265x JOURNAL
Characterization and Quantification of Isoprene-Derived Epoxydiols in Ambient Aerosol in the Southeastern United States
AUTHOR(S): Chan, Man Nin; Surratt, Jason D.; Claeys, Magda; Edgerton, Eric S.; Tanner, Roger L.; Shaw, Stephanie L.; Zheng, Mei; Knipping, Eladio M.; Eddingsaas, Nathan C.; Wennberg, Paul O.; Seinfeld, John H.
LOCATION: Division of Engineering and Applied Science, CaliforniaInstitute of Technology, Pasadena, CA, USA
JOURNAL: Environ. Sci. Technol. (Environmental Science & Technology)
DATE: 2010 VOLUME: 44 NUMBER: 12 PAGES: 4590-4596 CODEN: ESTHAG
ISSN: 0013-936X LANGUAGE: English PUBLISHER: American Chemical Society

4/3/12 (Item 2 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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151062209 CA: 151(3)62209v JOURNAL
Isoprene, Monoterpene, and Sesquiterpene Oxidation Products in the High Arctic Aerosols during Late Winter to Early Summer
AUTHOR(S): Fu, Pingqing; Kawamura, Kimitaka; Chen, Jing; Barrie, Leonard A.
LOCATION: Institute of Low Temperature Science, Hokkaido University, Sapporo, Japan, 060-0819
JOURNAL: Environ. Sci. Technol. (Environmental Science & Technology)
DATE: 2009 VOLUME: 43 NUMBER: 11 PAGES: 4022-4028 CODEN: ESTHAG
ISSN: 0013-936X LANGUAGE: English PUBLISHER: American Chemical Society

4/3/13 (Item 3 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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151013049 CA: 151(1)13049j JOURNAL
Validation and understanding of moderate resolution imaging spectroradiometer aerosol products (C5) using ground-based measurements from the handheld Sun photometer network in China
AUTHOR(S): Li, Zhanqing; Niu, Feng; Lee, Kwon-Ho; Xin, Jinyuan; Hao, Wei-Min; Nordgren, Bryce; Wang, Yuesi; Wang, Pucai

Samsung Bioepis Exhibit No. 1002
Page 8568

LOCATION: Department of Atmospheric and Oceanic Science, University of Maryland, College Park, MD, USA
JOURNAL: J. Geophys. Res., (Atmos.) (Journal of Geophysical Research, (Atmospheres)) DATE: 2007 VOLUME: 112 NUMBER: D22 PAGES: D22S07/1-D22S07/16, 2pl CODEN: JGRDE3 LANGUAGE: English PUBLISHER: American Geophysical Union

4/3/14 (Item 4 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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150570841 CA: 150(26)570841g PATENT
Concentrated fragrance composition provided in metered aerosol spray
INVENTOR(AUTHOR): Clark, Paul Alexander
LOCATION: USA
ASSIGNEE: S. C. Johnson & Son, Inc.
PATENT: PCT International ; WO 200967187 A1 DATE: 20090528
APPLICATION: WO 2008US12868 (20081118) *US 2007943051 (20071120)
PAGES: 34pp. CODEN: PIXXD2 LANGUAGE: English
PATENT CLASSIFICATIONS:

IPCR/8 + Level	Value	Position	Status	Version	Action	Source	Office:
A61L-0009/01	A	I	F	B	20060101	H	EP
A61L-0009/012	A	I	L	B	20060101	H	EP
A61L-0009/14	A	I	L	B	20060101	H	EP
B65D-0083/14	A	I	L	B	20060101	H	EP
C09K-0003/30	A	I	L	B	20060101	H	EP
A61L-0101/34	A	I	L	B	20060101	H	EP
A61L-0101/38	A	I	L	B	20060101	H	EP

DESIGNATED COUNTRIES: AE; AG; AL; AM; AO; AT; AU; AZ; BA; BB; BG; BH; BR; BW; BY; BZ; CA; CH; CN; CO; CR; CU; CZ; DE; DK; DM; DO; DZ; EC; EE; EG; ES; FI; GB; GD; GE; GH; GM; GT; HN; HR; HU; ID; IL; IN; IS; JP; KE; KG; KM; KN; KP; KR; KZ; LA; LC; LK; LR; LS; LT; LU; LY; MA; MD; ME; MG; MK; MN; MW; MX; MY; MZ; NA; NG; NI; NO; NZ; OM; PG; PH; PL; PT; RO; RS; RU; SC; SD; SE; SG; SK; SL; SM; ST; SV; SY; TJ; TM; TN; TR DESIGNATED REGIONAL: AT; BE; BG; CH ; CY; CZ; DE; DK; EE; ES; FI; FR; GB; GR; HR; HU; IE; IS; IT; LT; LU; LV; MC; MT; NL; NO; PL; PT; RO; SE; SI; SK; TR; BF; BJ; CF; CG; CI; CM; GA; GN; GQ; GW; ML; MR; NE; SN; TD; TG; BW; GH; GM; KE; LS; MW; MZ; NA; SD; SL; SZ; TZ; UG; ZM; ZW; AM; AZ; BY; KG; KZ; MD; RU; TJ; TM

4/3/15 (Item 5 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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149581349 CA: 149(26)581349h JOURNAL
Origin of C2-C5 dicarboxylic acids in the European atmosphere inferred from year-round aerosol study conducted at a west-east transect
AUTHOR(S): Legrand, M.; Preunkert, S.; Oliveira, T.; Pio, C. A.; Hammer, S.; Gelencser, A.; Kasper-Giebl, A.; Laj, P.
LOCATION: Laboratoire de Glaciologie et Geophysique de l'Environnement, Centre National de la Recherche Scientifique, St. Martin d'Heres, Fr.
JOURNAL: J. Geophys. Res., (Atmos.) (Journal of Geophysical Research, (Atmospheres)) DATE: 2007 VOLUME: 112 NUMBER: D23 PAGES: D23S07/1-D23S07/14 CODEN: JGRDE3 LANGUAGE: English PUBLISHER: American Geophysical Union

4/3/16 (Item 6 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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Samsung Bioepis Exhibit No. 1002
Page 8569

144191489 CA: 144(11)191489r PATENT
Aerosol compositions, devices and methods for animal feeding.
INVENTOR(AUTHOR): Deem, Gary; Sheppard, Don; Sheppard, Greg
LOCATION: USA
ASSIGNEE: Grain Processing Corporation
PATENT: U.S. Pat. Appl. Publ. ; US 20060034978 A1 DATE: 20060216
APPLICATION: US 2004918895 (20040816)
PAGES: 8 pp. CODEN: USXXCO LANGUAGE: English
PATENT CLASSIFICATIONS:
CLASS: 426116000
IPCR/8 + Level Value Position Status Version Action Source Office:
A23L-0001/00 A I F B 20060101 20060216 H US

4/3/17 (Item 7 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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142356740 CA: 142(19)356740q PATENT
Aerosol coating compositions containing alcohol solvents and their products packaged in spray cans
INVENTOR(AUTHOR): Hashizume, Ikuya; Yamada, Akira; Ogawa, Shingo; Inoue, Tadaaki; Nonogaki, Sadamu
LOCATION: Japan,
ASSIGNEE: Suzuka Fine Co., Ltd.; C.I. Chemical Y. K.; Wide Co., Ltd.
PATENT: Japan Kokai Tokkyo Koho ; JP 200597391 A2 DATE: 20050414
APPLICATION: JP 2003331350 (20030924)
PAGES: 5 pp. CODEN: JKXXAF LANGUAGE: Japanese
PATENT CLASSIFICATIONS:
CLASS: C09D-201/00A; C09D-007/12B

4/3/18 (Item 8 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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142027041 CA: 142(2)27041b JOURNAL
Low-Molecular-Weight and Oligomeric Components in Secondary Organic Aerosol from the Ozonolysis of Cycloalkenes and α-Pinene
AUTHOR(S): Gao, Song; Keywood, Melita; Ng, Nga L.; Surratt, Jason; Varutbangkul, Varuntida; Bahreini, Roya; Flagan, Richard C.; Seinfeld, John H.
LOCATION: Departments of Environmental Science and Engineering and Chemical Engineering, California Institute of Technology, Pasadena, CA, 91125, USA
JOURNAL: J. Phys. Chem. A (Journal of Physical Chemistry A) DATE: 2004
VOLUME: 108 NUMBER: 46 PAGES: 10147-10164 CODEN: JPCAFH ISSN: 1089-5639 PUBLISHER ITEM IDENTIFIER: 1089-5639(04)07466-3 LANGUAGE: English PUBLISHER: American Chemical Society

4/3/19 (Item 9 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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136107545 CA: 136(7)107545k PATENT
Pharmaceutical aerosol formulations with two or more active substances
INVENTOR(AUTHOR): McNamara, Daniel P.; Destefano, George A.
LOCATION: USA
PATENT: U.S. Pat. Appl. Publ. ; US 20020006384 A1 DATE: 20020117

Samsung Bioepis Exhibit No. 1002
Page 8570

APPLICATION: US 328073 (19990608)
PAGES: 4 pp. CODEN: USXXCO LANGUAGE: English
PATENT CLASSIFICATIONS:
CLASS: 424046000; A61L-009/04A; A61K-009/14B

4/3/20 (Item 10 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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133060442 CA: 133(5)60442v PATENT
Aerosol-type water-thinned cleaning composition with good detergency and sudsing stability
INVENTOR(AUTHOR): Kishi, Minoru; Yamakishi, Hiroshi; Sonoda, Akiko; Sakaki, Takako
LOCATION: Japan,
ASSIGNEE: Lion Corp.
PATENT: Japan Kokai Tokkyo Koho ; JP 2000178587 A2 DATE: 20000627
APPLICATION: JP 98359534 (19981217)
PAGES: 13 pp. CODEN: JKXXAF LANGUAGE: Japanese
PATENT CLASSIFICATIONS:
CLASS: C11D-001/74A; C11D-003/18B; C11D-003/20B; C11D-017/08B

4/3/21 (Item 11 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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130126256 CA: 130(10)126256j PATENT
Spot removal compositions for use in aerosol dispensers
INVENTOR(AUTHOR): De Jager, Jan
LOCATION: USA
ASSIGNEE: S.C. Johnson + Son, Inc.
PATENT: European Pat. Appl. ; EP 890670 A2 DATE: 19990113
APPLICATION: EP 98305491 (19980710) *GB 9714707 (19970711)
PAGES: 7 pp. CODEN: EPXXDW LANGUAGE: English
PATENT CLASSIFICATIONS:
CLASS: D06L-001/00A; C11D-017/00B; C11D-001/12B; C11D-003/18B; C11D-003/12B; C11D-003/06B; C11D-003/37B
DESIGNATED COUNTRIES: AT; BE; CH; DE; DK; ES; FR; GB; GR; IT; LI; LU; NL; SE; MC; PT; IE; SI; LT; LV; FI; RO

4/3/22 (Item 12 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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129199325 CA: 129(16)199325v PATENT
Insecticidal aerosol spray compositions characterized by the absence of a fragrance or masking agent
INVENTOR(AUTHOR): McLeod, Susan Donnaire
LOCATION: Australia
ASSIGNEE: R and C Products Pty. Limited
PATENT: Britain UK Pat. Appl. ; GB 2314774 A1 DATE: 19980114
APPLICATION: GB 9613909 (19960703)
PAGES: 14 pp. CODEN: BAXXDU LANGUAGE: English
PATENT CLASSIFICATIONS:
CLASS: A01N-025/06A

4/3/23 (Item 13 from file: 399)

Samsung Bioepis Exhibit No. 1002
Page 8571

DIALOG(R)File 399:CA SEARCH(R)
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126173416 CA: 126(13)173416p PATENT
Aerosol cleaning compositions containing hydrocarbon propellants
INVENTOR(AUTHOR): Silvester, Raymond Neville
LOCATION: Australia
ASSIGNEE: R & C Products Pty. Limited
PATENT: PCT International ; WO 9700934 A1 DATE: 19970109
APPLICATION: WO 96AU373 (19960621) *GB 9512900 (19950623)
PAGES: 29 pp. CODEN: PIXXD2 LANGUAGE: English
PATENT CLASSIFICATIONS:
CLASS: C11D-017/00A; C11D-003/18B
DESIGNATED COUNTRIES: AL; AM; AT; AU; AZ; BB; BG; BR; BY; CA; CH; CN; CZ; DE; DK; EE; ES; FI; GB; GE; HU; IS; JP; KE; KG; KP; KR; KZ; LK; LR; LS; LT; LU; LV; MD; MG; MK; MN; MW; MX; NO; NZ; PL; PT; RO; RU; SD; SE; SG; SI
DESIGNATED REGIONAL: KE; LS; MW; SD; SZ; UG; AT; BE; CH; DE; DK; ES; FI; FR; GB; GR; IE; IT; LU; MC; NL; PT; SE; BF; BJ; CF; CG; CI; CM; GA; GN; ML

4/3/24 (Item 14 from file: 399)
DIALOG(R)File 399:CA SEARCH(R)
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87106107 CA: 87(14)106107b CONFERENCE PROCEEDING
Measurements of photodegraded C2-C5 hydrocarbons from aerosol particulates collected in Houston, Texas
AUTHOR(S): Nahm, James J. W.; Pier, Stanley M.
LOCATION: Texas Air Control Board, Houston, Tex.
JOURNAL: Spec. Conf. Ozone/Oxid. - Interact. Total Environ., (Proc.)
DATE: 1976 PAGES: 157-65 CODEN: 35VPAO LANGUAGE: English PUBLISHER: Air Pollut. Control Assoc.,Pittsburgh, Pa
?

Samsung Bioepis Exhibit No. 1002
Page 8572

Search Notes	Application/Control No.	Applicant(s)/Patent Under Reexamination
	11127438	WANG, YI
	Examiner	Art Unit
	F. Pierre VanderVegt	1644

SEARCHED			
Class	Subclass	Date	Examiner

SEARCH NOTES		
Search Notes	Date	Examiner
PALM Inventor, 10/655,861, 60/408,571, 60/469,189 EAST, STN Medline Embase scisearch	8/26/08	PV
search strategy ATTACHED	8/28/08	PV
PALM related / priority USSNs	08/12/2010	PG
WEST / DIALOG	08/12/2010	PG

INTERFERENCE SEARCH			
Class	Subclass	Date	Examiner

	/Phillip Gambel/ Primary Examiner.Art Unit 1644

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

(Use as many sheets as necessary)

Sheet 1 of 1

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	F. P. Vandervegt
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
		WO98/18827	05-07-1998	Farmac Nederland B.V.		

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

Examiner Signature	/Phillip Gambel/	Date Considered	08/15/2010

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24173664_1.DOC

ALL REFERENCES CONSIDERED EXCEPT WHERE LINED THROUGH. /P.G./

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Search Results

Term	Documents
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ANTI-C5	186
ANTI-C5S	0
TREAT	192910
TREATS	24298
ASTHMA	37109
ASTHMAS	170
BRONCHITIS	12024
BRONCHITI	1
EMPHYSEMA	9651
((c5 or anti-c5)same(inhibit$ or block$ or antagoni$ or reduc$ or prevent$ or suppress$) and (therap$ or treat)same(asthma)same (bronchitis or emphysema)).PGPB.	164

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US Patents Full-Text Database
US Patents OCR Backfile
EPO Abstracts Database
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IBM Technical Disclosure Bulletin Database

Search Type: Prior Art Interference

Search: L17

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Recall Text Clear Interrupt

Search History

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Page 8575

Set Name Side by Side	Query	Hit Count	Set Name Result Set	Set Name Grid
Prior Art Searches				
	DB=PGPB; PLUR=YES; OP=ADJ			
L17	(c5 or anti-c5)same(inhibit$ or block$ or antagoni$ or reduc$ or prevent$ or suppress$) and (therap$ or treat)same(asthma) same(bronchitis or emphysema)	164	L17	L17
	DB=USPT; PLUR=YES; OP=ADJ			
L16	(c5 or anti-c5)same(inhibit$ or block$ or antagoni$ or reduc$ or prevent$ or suppress$) and (therap$ or treat)same(asthma) same(bronchitis or emphysema)	35	L16	L16
	DB=PGPB; PLUR=YES; OP=ADJ			
L15	(c5 or anti-c5)same(inhibit$ or block$ or antagoni$ or reduc$ or prevent$ or suppress$) and (therap$ or treat)same(asthma) same(bronchitis or emphysema)	164	L15	L15
L14	(anti-c5)same(antibod$) and (asthma or bronchitis or emphysema)	118	L14	L14
	DB=USPT; PLUR=YES; OP=ADJ			
L13	L12	22	L13	L13
	DB=PGPB,USPT; PLUR=YES; OP=ADJ			
L12	(anti-c5)same(antibod$) and (asthma or bronchitis or emphysema)	140	L12	L12
L11	(c5 or anti-c5)same(antibod$) same (asthma or bronchitis or emphysema)	89	L11	L11
	DB=EPAB,JPAB,DWPI; PLUR=YES; OP=ADJ			
L10	(c5 or anti-c5)same(antibod$) and (nebulizer or aerosol) and (asthma or bronchitis or emphysema)	3	L10	L10
	DB=USPT; PLUR=YES; OP=ADJ			
L9	(c5 or anti-c5)same(antibod$) and (nebulizer or aerosol) and (asthma or bronchitis or emphysema)	61	L9	L9
	DB=PGPB; PLUR=YES; OP=ADJ			
L8	(c5 or anti-c5)same(antibod$) and (nebulizer or aerosol) and (asthma or bronchitis or emphysema)	307	L8	L8
L7	L3 and (asthma)	136	L7	L7
	DB=USPT; PLUR=YES; OP=ADJ			
L6	L5	32	L6	L6
	DB=PGPB,USPT,EPAB,JPAB,DWPI; PLUR=YES; OP=ADJ			
L5	L3 and (asthma or bonchitis or emphysema)	173	L5	L5
L4	L3 and (athma or bonchitis or emphysema)	46	L4	L4

Samsung Bioepis Exhibit No. 1002
Page 8576

L3	L2 and (aerosol or nebulizer)	593	L3	L3
L2	L1 and (complement or c5 or anti-c5)	3941	L2	L2
L1	wang.in.	384566	L1	L1

END OF SEARCH HISTORY

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Page 8577

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Sheet 1 of 2

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	Phillip Gambel
Attorney Docket Number	ALXN-P02-102

U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number Number-Kind Code[2] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear

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Examiner Initials*	Cite No.[1]	Foreign Patent Document Country Code[3]-Number[4]-Kind Code[5] (if known)	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages Or Relevant Figures Appear	T[6]
	BD	CA 2 189 015	11-09-1995	Alexion Pharmaceuticals, Inc.		
	BE	CA 2 198 706	03-28-1996	Alexion Pharmaceuticals, Inc.		

Examiner Signature		Date Considered	

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INFORMATION DISCLOSURE STATEMENT BY APPLICANT

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Sheet 2 of 2

Complete if Known	
Application Number	11/127,438
Filing Date	May 11, 2005
First Named Inventor	Yi Wang
Art Unit	1644
Examiner Name	Phillip Gambel
Attorney Docket Number	ALXN-P02-102

NON PATENT LITERATURE DOCUMENTS

Examiner Initials*	Cite No.[1]	Include name of the author (in CAPITAL LETTERS), title of the article (when appropriate), title of the item (book, magazine, journal, serial, symposium, catalog, etc.), date, page(s), volume-issue number(s), publisher, city and/or country where published.	T[2]

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant. ** CITE NO.: Those document(s) which are marked with an double asterisk (**) next to the Cite No. are not supplied because they were previously cited by or submitted to the Office in a prior application relied upon in this application for an earlier filing date under 35 U.S.C. 120.

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Page 8579

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CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 1995/05/01
(87) Date publication PCT/PCT Publication Date: 1995/11/09
(45) Date de délivrance/Issue Date: 2010/04/13
(85) Entrée phase nationale/National Entry: 1996/10/28
(86) N° demande PCT/PCT Application No.: US 1995/005688
(87) N° publication PCT/PCT Publication No.: 1995/029697
(30) Priorité/Priority: 1994/05/02 (US08/236,208)

(51) Cl.Int./Int.Cl. *C12N 15/13* (2006.01), *A61K 39/395* (2006.01), *C07K 14/47* (2006.01), *C07K 16/18* (2006.01), *C12N 5/20* (2006.01), *G01N 33/53* (2006.01), *A61K 38/00* (2006.01)

(72) Inventeurs/Inventors:
EVANS, MARK J., US;
MATIS, LOUIS, US;
MUELLER, EILEEN, US;
NYE, STEVEN H., US;
ROLLINS, SCOTT, US;
...

(73) Propriétaire/Owner:
ALEXION PHARMACEUTICALS, INC., US

(74) Agent: MCFADDEN, FINCHAM

(54) Titre : PROCEDES ET COMPOSITIONS S'APPLIQUANT AU TRAITEMENT DE LA GLOMERULONEPHRITE ET D'AUTRES MALADIES INFLAMMATOIRES

(54) Title: METHODS AND COMPOSITIONS FOR THE TREATMENT OF GLOMERULONEPHRITIS AND OTHER INFLAMMATORY DISEASES

(57) Abrégé/Abstract:
The use of anti-C5 antibodies, e.g., monoclonal antibodies, to treat glomerulonephritis (GN) is disclosed. The administration of such antibodies at low dosage levels has been found to significantly reduce glomerular inflammation/enlargement and other pathologic conditions associated with GN. Also disclosed are novel anti-C5 antibodies and anti-C5 antibody-encoding nucleic acid molecules. These antibodies are useful in the treatment of GN and other inflammatory conditions involving pathologic activation of the complement system.

CA 2189015 C 2010/04/13
(11)(21) 2 189 015
(13) C

(72) **Inventeurs(suite)/Inventors(continued):** ROTHER, RUSSELL P., US; SPRINGHORN, JEREMY P., US; SQUINTO, STEPHEN P., US; THOMAS, THOMAS C., US; WANG, YI, US; WILKINS, JAMES A., US

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Page 8581

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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/36, 39/00, 39/395, C07K 14/00, 14/75, 16/00, 16/18, 16/36, 16/46, C12N 5/10, 5/20, 15/09, 15/10, 15/13, 15/63, C12P 21 /02, 21 /08

A1

(11) International Publication Number: **WO 95/29697**

(43) International Publication Date: 9 November 1995 (09.11.95)

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(22) International Filing Date: 1 May 1995 (01.05.95)

(30) Priority Data:
08/236,208 2 May 1994 (02.05.94) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/236,208 (CIP)
Filed on 2 May 1994 (02.05.94)

(71) Applicant *(for all designated States except US)*: ALEXION PHARMACEUTICALS, INC. [US/US]; Suite 360, 25 Science Park, New Haven, CT 06511 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: EVANS, Mark, J. [US/US]; 528 Wood Hill Road, Cheshire, CT 06410 (US). MATIS, Louis [US/US]; 775 Flintlock Road, Southport, CT 06490 (US). MUELLER, Eileen, Elliott [US/US]; 30 Silver Sands Road - Unit 19F, East Haven, CT 06512 (US). NYE, Steven, H. [US/US]; 6 Valley Run Drive, Cromwell, CT 06416 (US). ROLLINS, Scott [US/US]; 12 Nutmeg Circle, Monroe, CT 06468 (US). ROTHER, Russell, P. [US/US]; 384 Black's Road, Cheshire, CT 06410 (US). SPRINGHORN, Jeremy, P. [US/US]; 105 Greens Loop, Cheshire, CT 06410 (US). SQUINTO, Stephen, P. [US/US]; 16 Coachmans Lane, Bethany, CT 06524 (US). THOMAS, Thomas, C. [US/US]; 23 Riverside Terrace, Madison, CT 06443 (US). WANG, Yi [CN/US]; 270 Sylvan Valley Road, Orange, CT 06477 (US). WILKINS, James, A. [US/US]; 21 Clark Road, Woodbridge, CT 06525 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR THE TREATMENT OF GLOMERULONEPHRITIS AND OTHER INFLAMMATORY DISEASES

(57) Abstract

The use of anti-C5 antibodies, e.g., monoclonal antibodies, to treat glomerulonephritis (GN) is disclosed. The administration of such antibodies at low dosage levels has been found to significantly reduce glomerular inflammation/enlargement and other pathologic conditions associated with GN. Also disclosed are novel anti-C5 antibodies and anti-C5 antibody-encoding nucleic acid molecules. These antibodies are useful in the treatment of GN and other inflammatory conditions involving pathologic activation of the complement system.

Samsung Bioepis Exhibit No. 1002
Page 8582

CLAIMS:

1. Use of an antibody that binds to complement component C5 in a patient's bloodstream in an amount effective to substantially reduce the cell-lysing ability of the complement present for the treatment of pre-existing glomerulonephritis.

2. The use of claim 1, wherein the antibody reduces the conversion of complement component C5 into complement components C5a and C5b.

3. The use of claim 1, wherein the antibody binds to C5b.

4. The use of claim 1, wherein the antibody does not substantially inhibit formation of complement component C3b.

5. The use of claim 1, wherein the antibody is in a dose that is not greater than 0.1 grams per kilogram.

6. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises an antibody to complement component C5, said antibody being effective in substantially reducing the cell-lysing ability of complement present in the patient's blood; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of pre-existing kidney disease.

7. The article of manufacture of claim 6, wherein the label indicates that said pharmaceutical agent is for use in the treatment of nephritis.

8. The article of manufacture of claim 7, wherein the label indicates that said pharmaceutical agent is for use in the treatment of glomerulonephritis.

9. The article of manufacture of claim 6, wherein the pharmaceutical agent is to be used at a dosage level not greater than 0.1 grams per kilogram.

10. An antibody comprising at least one antibody-antigen binding site, said antibody exhibiting specific binding to human complement component C5, said specific binding being targeted to the alpha chain of human complement component C5, wherein the antibody inhibits complement activation in a human body fluid and does not specifically bind to the human complement activation product free C5a.

11. The antibody of claim 10, wherein the inhibition of complement activation in the human body fluid is measurable as a substantial increment of blockade of C5a generation and a substantial increment of blockade of complement hemolytic activity in the body fluid, said increment of blockade of C5a generation being substantially

Samsung Bioepis Exhibit No. 1002
Page 8583

equal to said increment of blockade of complement hemolytic activity.

12. The antibody of claim 10, wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C3.

13. The antibody of claim 10, wherein, upon binding to human C5, the antibody substantially inhibits the ability of C5 to bind to human complement component C4.

14. The antibody of claim 10, wherein the antibody binds specifically with a peptide comprising amino acid residues 660-1019 of SEQ ID NO:2.

15. The antibody of claim 10, wherein the antibody binds specifically to a peptide comprising amino acid residues 725-1049 of SEQ ID NO:2.

16. The antibody of claim 10, wherein the antibody binds specifically to a peptide comprising amino acid residues 850-1049 of SEQ ID NO:2.

17. The antibody of claim 10, wherein the antibody binds specifically to a peptide comprising amino acid residues 1-21 of SEQ ID NO:1.

18. The antibody of claim 10, wherein the inhibition of complement activation in the human body fluid is measurable as a substantially complete blockade of C5a generation in the body fluid and a substantially complete blockade of complement hemolytic activity in the body fluid when the antibody is added to the body fluid at a concentration yielding a ratio equal to or less than 10 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

19. The antibody of claim 18, wherein the concentration yields a ratio equal to or less than 3 moles of antibody-antigen binding sites of the antibody to 1 mole of human C5 in the body fluid.

20. Hybridoma 5G1.1 having ATCC designation HB-11625.

21. An antibody produced by the hybridoma of claim 20.

22. A nucleic acid molecule comprising a nucleotide sequence encoding a scFv polypeptide comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:7 which binds to the α-chain of C5.

23. A nucleic acid molecule comprising a nucleotide sequence encoding a light chain of an antibody, wherein said antibody binds to the α-chain of C5, wherein a light chain variable region of said antibody comprises amino acid 3 through amino acid 110 of SEQ ID NO: 9.

24. A nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain of an antibody, wherein said antibody binds to the α-chain of C5, wherein

Samsung Bioepis Exhibit No. 1002
Page 8584

a heavy chain variable region of said antibody comprises amino acid 1 through amino acid 122 of SEQ ID NO: 10.

25. An isolated protein which binds to the α-chain of C5 comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:9; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:10.

26. An isolated polypeptide comprising an amino acid sequence encoded by the nucleic acid molecule of claim 22, wherein the polypeptide is an antibody which binds to C5.

27. An isolated polypeptide which binds to the α-chain of C5, wherein said polypeptide comprises: i) a variable light chain region encoded by the nucleic acid of claim 23 and ii) a variable heavy chain region encoded by the nucleic acid of claim 24.

28. A nucleic acid vector comprising a nucleic acid molecule covalently and operatively linked to a promoter so that a host containing the vector expresses the polypeptide coded for by the nucleic acid molecule, wherein the nucleic acid molecule is the nucleic acid molecule of claim 22, claim 23 or claim 24.

29. A recombinant host cell containing the nucleic acid vector of claim 28.

30. A method for producing an isolated C5 antibody polypeptide comprising:

(a) growing a recombinant host cell containing a nucleic acid vector comprising a nucleic acid molecule covalently and operatively linked to a promoter so that a host containing the vector expresses the polypeptide coded for by the nucleic acid molecule, wherein the nucleic acid molecule comprises i) the nucleic acid molecule of claim 22 or ii) the nucleic acid molecules of claims 23 and 24, such that the polypeptide is expressed by the host cell; and

(b) isolating the expressed polypeptide, wherein the expressed polypeptide is an anti-C5 antibody.

31. The isolated anti-C5 antibody produced by the method of claim 30.

32. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:8, which binds to the α-chain of C5.

Samsung Bioepis Exhibit No. 1002
Page 8585

33. A nucleic acid molecule comprising a nucleotide sequence encoding an scFv comprising an amino acid sequence corresponding to amino acid 1 through amino acid 248 of SEQ ID NO:17, which binds to the α-chain of C5.

34. A nucleic acid molecule comprising a nucleotide sequence encoding a light chain of an antibody, wherein said antibody binds to the α-chain of C5, wherein a light chain variable region of said antibody comprises amino acid 1 through amino acid 108 of SEQ ID NO:15.

35. A nucleic acid molecule comprising a nucleotide sequence encoding a light chain of an antibody, wherein said antibody binds to the α-chain of C5, wherein a light chain variable region of said antibody comprises amino acid 3 through amino acid 110 of SEQ ID NO:14.

36. A nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain of an antibody, wherein said antibody binds to the α-chain of C5, wherein a heavy chain variable region of said antibody comprises amino acid 1 through amino acid 122 of SEQ ID NO:16.

37. A nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain of an antibody, wherein said antibody binds to the α-chain of C5, wherein a heavy chain variable region of said antibody comprises amino acid 1 through amino acid 122 of SEQ ID NO:12.

38. A nucleic acid molecule comprising a nucleotide sequence encoding a heavy chain of an antibody, wherein said antibody binds to the α-chain of C5, wherein a heavy chain variable region of said antibody comprises amino acid 1 through amino acid 122 of SEQ ID NO:11.

39. An isolated protein which binds to the α-chain of C5 comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

40. An isolated protein which binds to the α-chain of C5 comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

Samsung Bioepis Exhibit No. 1002
Page 8586

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

41. An isolated protein which binds to the α-chain of C5 comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 1 through amino acid 108 of SEQ ID NO:15; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

42. An isolated protein which binds to the α-chain of C5 comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:16.

43. An isolated protein which binds to the α-chain of C5 comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:12.

44. An isolated protein which binds to the α-chain of C5 comprising:

(a) a first polypeptide region comprising a variable light chain region amino acid sequence corresponding to amino acid 3 through amino acid 110 of SEQ ID NO:14; and

(b) a second polypeptide region comprising a variable heavy chain region amino acid sequence corresponding to amino acid 1 through amino acid 122 of SEQ ID NO:11.

45. An isolated protein comprising:

i) a light chain variable region comprising a light chain CDR1, a light chain CDR2, and a light chain CDR3, and

Samsung Bioepis Exhibit No. 1002
Page 8587

ii) a heavy chain variable region comprising a heavy chain CDR1, a heavy chain CDR2 and a heavy chain CDR3,

wherein said protein comprises the amino acid sequence encoded by a) the nucleic acid molecule of claim 32 or 33 or b) a nucleic acid molecule of 1) claim 34 or 35 and 2) a nucleic acid molecule of claim 36, 37 or 38,

wherein the isolated protein is an anti-C5 antibody.

46. A nucleic acid vector comprising a nucleic acid molecule covalently and operatively linked to a promoter so that a host containing the vector expresses the polypeptide coded for by the nucleic acid molecule, wherein the nucleic acid molecule is the nucleic acid molecule of claim 32, claim 33, claim 34, claim 35, claim 36, claim 37 or claim 38.

47. A recombinant host cell containing the nucleic acid vector of claim 46.

48. A method for producing an isolated anti-C5 antibody protein comprising growing a recombinant host cell containing a nucleic acid vector comprising a nucleic acid molecule covalently and operatively linked to a promoter so that a host containing the vector expresses the polypeptide coded for by the nucleic acid molecule, wherein the nucleic acid molecule is the nucleic acid molecule of claim 32 or 33.

49. A method for producing an isolated anti-C5 antibody protein comprising growing a recombinant host cell containing:

i) a nucleic acid vector comprising a nucleic acid molecule covalently and operatively linked to a promoter so that a host containing the vector expresses the polypeptide coded for by the nucleic acid molecule, wherein the nucleic acid molecule is the nucleic acid molecule of claim 34 or 35 which encodes a variable light chain; and

ii) a nucleic acid vector comprising a nucleic acid molecule covalently and operatively linked to a promoter so that a host containing the vector expresses the polypeptide coded for by the nucleic acid molecule, wherein the nucleic acid molecule is the nucleic molecule of claim 36, 37 or 38 which encodes a variable heavy chain,

wherein said variable heavy chain is capable of combining with said variable light chain to form said antibody.

50. The isolated anti-C5 antibody produced by the method of any one of claims 48 or 49.

51. An isolated nucleic acid molecule encoding a light chain of an antibody, wherein said antibody binds to the α-chain of C5, comprising a nucleotide sequence encoding i) a variable light region CDR1 comprising an amino acid sequence

Samsung Bioepis Exhibit No. 1002
Page 8588

corresponding to amino acid 26 through amino acid 36 of SEQ ID NO:8, ii) a variable light region CDR2 comprising an amino acid sequence corresponding to amino acid 52 through amino acid 58 of SEQ ID NO:8, and iii) a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8.

52. An isolated nucleic acid molecule encoding a heavy chain of an antibody, wherein said antibody binds to the α-chain of C5, comprising i) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8; ii) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; and iii) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

53. An isolated nucleic acid molecule encoding a heavy chain of an antibody, wherein said antibody binds to the α-chain of C5, comprising i) a nucleotide sequence encoding a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 157 through amino acid 161 of SEQ ID NO:8; ii) a nucleotide sequence encoding a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; and iii) a nucleotide sequence encoding a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

54. An isolated nucleic acid molecule, the encoded protein of which binds to the α-chain of C5, comprising:

(a) a nucleotide sequence encoding i) a variable light region comprising a variable light chain CDR1 comprising an amino acid sequence corresponding to amino acid 26 through amino acid 36 of SEQ ID NO:8; ii) a variable light chain CDR2 comprising an amino acid sequence corresponding to amino acid 52 through amino acid 58 of SEQ ID NO:8; and iii) a variable light chain CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8; and

(b) a nucleotide sequence encoding i) a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8; ii) a variable heavy region CDR2 comprising an amino acid sequence

Samsung Bioepis Exhibit No. 1002
Page 8589

corresponding to amino acid 176 through amino acid 186 of SEQ ID NO:8; and iii) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

55. An isolated nucleic acid molecule, the encoded protein of which binds to the α-chain of C5 comprising:

(a) a nucleotide sequence encoding i) a variable light region comprising a variable light chain CDR1 comprising an amino acid sequence corresponding to amino acid 26 through amino acid 36 of SEQ ID NO:8; ii) a variable light chain CDR2 comprising an amino acid sequence corresponding to amino acid 52 through amino acid 58 of SEQ ID NO:8; and iii) a variable light chain CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8; and

(b) a nucleotide sequence encoding i) a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8; ii) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; and iii) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

56. An isolated nucleic acid molecule, the encoded protein of which binds to the α-chain of C5 comprising:

(a) a nucleotide sequence encoding i) a variable light region comprising a variable light chain CDR1 comprising an amino acid sequence corresponding to amino acid 26 through amino acid 36 of SEQ ID NO:8; ii) a variable light chain CDR2 comprising an amino acid sequence corresponding to amino acid 52 through amino acid 58 of SEQ ID NO:8; and iii) a variable light chain CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8; and

(b) a nucleotide sequence encoding (i) a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8; (ii) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 179 through amino acid 182 of SEQ ID NO:7; and (iii) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

57. The antibody of claim 10, wherein the antibody is a recombinant antibody that comprises a human constant domain.

Samsung Bioepis Exhibit No. 1002
Page 8590

58. An isolated protein which binds to the α-chain of C5, wherein said protein comprises a light chain CDR1, a light chain CDR2, a light chain CDR3, a heavy chain CDR1, a heavy chain CDR2, and a heavy chain CDR3, wherein said protein comprises the amino acid sequence encoded by the nucleic acid molecule of claim 54, 55 or 56.

59. An isolated nucleic acid molecule, the encoded protein of which binds to the α-chain of C5, comprising:

(a) a nucleotide sequence encoding i) a variable light region comprising a variable light region CDR1 comprising an amino acid sequence corresponding to amino acid 26 through amino acid 36 of SEQ ID NO:8; ii) a variable light region CDR2 comprising an amino acid sequence corresponding to amino acid 52 through amino acid 58 of SEQ ID NO:8; iii) a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8; and

(b) a nucleotide sequence encoding i) a variable heavy region comprising a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 157 through amino acid 161 of SEQ ID NO:8; ii) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; iii) a variable heavy region CDR3 comprising an amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

60. An isolated nucleic acid molecule, the encoded protein of which binds to the α-chain of C5, comprising:

(a) a nucleotide sequence encoding i) a variable light region comprising a variable light region CDR1 comprising an amino acid sequence corresponding to amino acid 26 through amino acid 36 of SEQ ID NO:8; ii) a variable light region CDR2 comprising an amino acid sequence corresponding to amino acid 52 through amino acid 58 of SEQ ID NO:8; iii) a variable light region CDR3 comprising an amino acid sequence corresponding to amino acid 91 through amino acid 99 of SEQ ID NO:8; and

(b) a nucleotide sequence encoding i) a variable heavy region comprising a variable heavy region CDR1 comprising an amino acid sequence corresponding to amino acid 152 through amino acid 161 of SEQ ID NO:8; ii) a variable heavy region CDR2 comprising an amino acid sequence corresponding to amino acid 176 through amino acid 192 of SEQ ID NO:8; iii) a variable heavy region CDR3 comprising an

Samsung Bioepis Exhibit No. 1002
Page 8591

amino acid sequence corresponding to amino acid 225 through amino acid 237 of SEQ ID NO:8.

61. The isolated protein of claim 58, wherein the protein is an anti-C5 antibody.

62. The nucleic acid molecule of claim 59 or 60, wherein the encoded protein is an anti-C5 antibody.

63. A nucleic acid vector comprising a nucleic acid molecule, said nucleic acid molecule corresponding to the nucleic acid molecule of claim 51, 52, 53, 59 or 60 covalently and operatively linked to a promoter so that a host containing the vector expresses the protein encoded by the nucleic acid molecule.

64. A recombinant host cell containing the nucleic acid vector of claim 63.

65. A method for producing an anti-C5 antibody comprising:

a) growing a recombinant host cell comprising the nucleic acid of claim 54, 55, 56, 59 or 60, wherein the variable light chain and variable heavy chain are expressed by the host cell and combine to form a protein, and

b) isolating the protein, wherein the expressed protein is an anti-C5 antibody.

66. A method for producing an anti-C5 antibody comprising:

a) growing a recombinant host cell comprising:

i) a nucleic acid encoding a variable light chain comprising a) a light chain CDR1 of amino acid 26 through amino acid 36 of SEQ ID NO:8; b) a light chain CDR2 of amino acid 52 through amino acid 58 of SEQ ID NO:8; and c) a light chain CDR3 of amino acid 91 through amino acid 99 of SEQ ID NO:8; and

ii) a nucleic acid encoding a variable heavy chain comprising a) a heavy chain CDR1 of amino acid 157 through amino acid 161 of SEQ ID NO:8; b) a heavy chain CDR2 of amino acid 176 through amino acid 192 of SEQ ID NO:8; and c) a heavy chain CDR3 of amino acid 225 through amino acid 237 of SEQ ID NO:8,

wherein the variable light chain and variable heavy chain are expressed by the host cell and combine to form a protein; and

b) isolating the protein, wherein the expressed protein is an anti-C5 antibody.

67. A method for producing an anti-C5 antibody comprising:

a) growing a recombinant host cell comprising:

Samsung Bioepis Exhibit No. 1002
Page 8592

i) a nucleic acid encoding a variable light chain comprising a) a light chain CDR1 of amino acid 26 through amino acid 36 of SEQ ID NO:8; b) a light chain CDR2 of amino acid 52 through amino acid 58 of SEQ ID NO:8; and c) a light chain CDR3 of amino acid 91 through amino acid 99 of SEQ ID NO:8; and

ii) a nucleic acid encoding a variable heavy chain comprising a) a heavy chain CDR1 of amino acid 152 through amino acid 161 of SEQ ID NO:8; b) a heavy chain CDR2 of amino acid 176 through amino acid 192 of SEQ ID NO:8; and c) a heavy chain CDR3 of amino acid 225 through amino acid 237 of SEQ ID NO:8,

wherein the variable light chain and variable heavy chain are expressed by the host cell and combine to form a protein; and

b) isolating the protein, wherein the expressed protein is an anti-C5 antibody.

68. The anti-C5 antibody produced by the method of claim 65.

69 Use of the antibody of claim 10, claim 21, claim 26, claim 31, claim 45, claim 50, claim 61 or claim 68 to substantially reduce hemolytic activity in a body fluid of a patient.

Samsung Bioepis Exhibit No. 1002
Page 8593

METHODS AND COMPOSITIONS FOR THE TREATMENT OF GLOMERULONEPHRITIS AND OTHER INFLAMMATORY DISEASES

FIELD OF THE INVENTION

The present invention relates to the treatment of glomerulonephritis (GN) and other inflammatory diseases, and more generally to therapeutic treatments involving the pharmacologic inhibition of a patient's complement system. In particular, the invention relates to the use of antibodies specific to human complement component C5 to accomplish such therapeutic treatment. The invention also relates to compositions comprising native monoclonal antibodies (mAbs) specific to human complement component C5 that block complement hemolytic activity and C5a generation at concentrations that substantially reach the theoretical one to two stoichiometric limit of antibody to antigen that can be achieved by a bivalent antibody. The invention further provides recombinant mAbs that are derivatives (including monovalent derivatives) of these native mAbs that provide substantially the same blocking activities as the native mAbs.

BACKGROUND OF THE INVENTION

I. Immune Complex Mediated Disease

The formation of immune complexes is the typical consequence of the interaction of antigens with specific antibodies. The inflammatory response that ensues when such complexes accumulate in a limited area is an important element of normal host defenses, leading to immune complex clearance and antigen destruction by phagocytic cells. In contrast, immune complex diseases are reflections of excess complex formation or retarded clearance, usually under conditions of exceptional antigen challenge or immunologic dysregulation. Under such circumstances, immune complexes are deposited or formed at specific tissue sites and resulting inflammatory responses lead to disease states due to localized or systemic tissue damage. The kidney, and more specifically the kidney structure known as the glomerulus, is a particularly important

Samsung Bioepis Exhibit No. 1002
Page 8594

site of immune complex deposition resulting in the development of serious disease conditions.

Human studies, and studies using animal models of human diseases, have implicated the complement system in the pathologies associated with a number of immune complex associated disorders. The activation of complement that mediates the pathology associated with these disorders may be a consequence of an autoimmune mechanism, or can be non-immunologic in origin.

The hypersensitivity response that occurs when antibodies bind to antigens either in tissues or in the circulation results from the activation of complement and the release of molecules that mediate inflammation. This process is classified as either being mediated by the binding of antibody to fixed tissue or cell bound antigens (Type II hypersensitivity) or to circulating antigens, resulting in the formation of circulating immune complexes and their subsequent pathogenic deposition in tissues (Type III hypersensitivity).

Type II hypersensitivity is mediated through the activation of complement following the binding of antibodies to fixed tissue antigens. The inflammatory response that ensues results from the activation of the proinflammatory and lytic components of the complement system and the subsequent recruitment of stimulated leukocytes to the sites of immune complex formation. The increased vascular permeability that results from the anaphylatoxic activities of C3a and C5a further enhances immune complex deposition and leukocyte recruitment.

The cross-linking of antibody bound cells or tissues to effector cells such as neutrophils, platelets, NK cells, and monocytes via their Fc receptors also plays a proinflammatory role. Such cross-linking activates effector cells, stimulating the release of oxygen radicals, prostaglandins, and leukotrienes, which release is further potentiated by the actions of activated complement components.

Examples of Type II hypersensitivity-mediated conditions include hyperacute rejection of transplanted organs, autoimmune hemolytic and thrombocytopenic states,

Samsung Bioepis Exhibit No. 1002
Page 8595

Goodpasture's syndrome (and associated glomerulonephritis and pulmonary hemorrhage), myasthenia gravis, pathologic sequellae associated with insulin-dependent diabetes melitus, and pemphigus vulgaris.

Type III hypersensitivity reactions involving circulating antigens can also result in the development of numerous pathologic conditions. These include glomerulonephritis (discussed in detail below), vasculitis (a potentially life-threatening inflammatory condition of large and/or small blood vessels), rheumatoid arthritis, dermatitis, and other disorders.

Other diseases associated with type III hypersensitivity reactions include autoimmune diseases such as systemic lupus erythematosis (SLE), many infectious diseases, neoplastic diseases, and a wide variety of other conditions (Dixon, et al. Immune Complex Injury, in Samter, (ed.) Immunological Diseases, 4th ed. Little Brown & Co. Boston, 1987).

II. Glomerulonephritis

The glomerulus is a key structural and functional element of the kidney. Each glomerulus is found as part of a larger structure that serves as the main functional unit of the kidney and is called a nephron. About a million nephrons are found in each kidney. Each glomerulus is a network of up to fifty parallel capillaries encased in a structure known as Bowman's capsule. The area inside Bowman's capsule that is not taken up by the glomerular capillaries is known as Bowman's space. The glomerulus functions as a filter, separating water and certain solutes from the proteins and cells of the blood into Bowman's space for further processing in the convoluted tubules, loop of Henle, and collecting duct of the nephron.

Glomerulonephritis (GN) is a disease of the kidney characterized by inflammation and resulting enlargement of the glomeruli that is typically due to immune complex formation. The accumulation of immune complexes in the glomeruli results in inflammatory responses, involving inter alia hypercellularity, that can cause total or partial blockage of the glomerulus through, among other factors, narrowing of

Samsung Bioepis Exhibit No. 1002
Page 8596

capillary lumens. One result of this process is the inhibition of the normal filtration function of the glomerulus. Blockage may occur in large numbers of glomeruli, directly compromising kidney function and often causing the abnormal deposition of proteins in the walls of the capillaries making up the glomerulus. Such deposition can, in turn, cause damage to glomerular basement membranes. Those glomeruli that are not blocked develop increased permeability, allowing large amounts of protein to pass into the urine, a condition referred to as proteinuria.

In many cases of severe GN, pathological structures called crescents are formed within the Bowman's space, further impeding glomerular filtration. These structures can only be seen by microscopic examination of tissue samples obtained by biopsy or necropsy, and are thus not always observed in those patients in which they occur. Crescents are a manifestation of hypercellularity and are thought to arise from the extensive abnormal proliferation of parietal epithelial cells, the cells that form the inner lining of the Bowman's capsule. Clinical research has shown that there is a rough correlation between the percentage of glomeruli with crescents and the clinical severity of the disease, and thus the patient's prognosis. When present in large numbers, crescents are a poor prognostic sign.

Symptoms of GN include: proteinuria; reduced glomerular filtration rate (GFR); serum electrolyte changes including azotemia (uremia, excessive blood urea nitrogen - BUN) and salt retention, leading to water retention resulting in hypertension and edema; hematuria and abnormal urinary sediments including red cell casts; hypoalbuminemia; hyperlipidemia; and lipiduria.

In 1990, over 210,000 patients in the United States required hemodialysis or transplantation for chronic renal failure at an annual cost in excess of 7 billion dollars, according to the United States Renal Data System (USRDS). The USRDS compiles data on kidney disease in the United States in conjunction with the National Institute of Diabetes and Digestive and Kidney Diseases, Division of Kidney, Urologic,

Samsung Bioepis Exhibit No. 1002
Page 8597

and Hematologic Diseases, of the National Institutes of Health (NIDDKD). The USRDS estimates that the costs of treatment for renal failure are now increasing by 20 percent annually.

GN is the third leading cause of death in end-stage renal disease patients, exceeded only by diabetes and hypertension. As a result, there is a clear and long felt need in the medical community for effective treatments for this condition. Research aimed at the development of new treatments for GN is ongoing worldwide. In the United States, the NIDDKD, the National Kidney Foundation, and several other public and private organizations sponsor research in this area. The National Kidney Foundation alone supplies over two million dollars annually to fund the efforts of kidney researchers.

III. Current Treatments for GN

Corticosteroid administration, typically as high doses of "pulse" intravenous methylprednisolone or oral prednisone therapy, is currently considered the most effective pharmacologic agent available for the treatment of GN. Such steroid therapy is often administered in combination with cytotoxic general immunosuppressive agents such as azathioprine or cyclophosphamide. The overall immune suppression and resulting increased susceptibility to infection, along with other debilitating side effects associated with both steroid and cytotoxic drug administration, limit the effective use of these drugs.

Aspirin-like non-steroidal anti-inflammatory drugs (NSAIDs) have also been used to reduce the glomerular inflammation and enlargement of GN. These drugs are not routinely used for this purpose, however, probably because of their relatively weak anti-inflammatory effects and propensity to cause gastrointestinal and other side effects in many patients.

The administration of anticoagulants such as heparin or warfarin sodium, and antithrombotic agents such as cyproheptadine, dipyridamole, or sulfinpyrazone, has been used on the basis of evidence suggesting the involvement of the coagulation process in the genesis of glomerular crescents. However, objective evidence of benefit from such therapies in

Samsung Bioepis Exhibit No. 1002
Page 8598

animals afflicted with experimentally induced crescentic GN has been inconsistent. Also, anticoagulants are dangerous drugs, as they can potentiate life-threatening bleeding episodes. They are especially hazardous in this regard in patients with advanced renal failure.

In addition to pharmacologic approaches, intensive plasma exchange (plasmapheresis) of 2 to 4 liters of plasma daily (or in some cases three times a week) can dramatically reduce high levels of circulating immune complexes when acute intervention in the inflammatory process is needed. Such treatment is expensive and requires that the patient be connected to the plasmapheresis machine for many hours each week. In addition, all procedures in which blood is removed from and returned to a patient are associated with an increased risk of infection. Nonetheless, plasma exchange is currently considered the most effective non-pharmacological treatment for removal of circulating immune complexes which can cause GN.

Circulating immune complex levels can also be decreased by eliminating or reducing the source of the antigen or antigens contained in the complexes by, for example, effective therapy of an underlying infection or change in an antibiotic. However, while such therapy is almost always a treatment of choice, great care must be taken since reduction of the antigen load alters the molar ratio of antigen to antibody involved in forming immune complexes and thus a dangerous temporary exacerbation of the inflammatory process may occur (see discussion below in Background Physiology & Pathology).

IV. Antibody Engineering

Native antibodies are multi-subunit animal protein molecules with highly specific antigen-binding properties. Animals make multiple classes of antibodies. There are five major classes (IgA, IgD, IgE, IgG and IgM) and a variety of subclasses. Native antibodies are made up of two or more heterodimeric subunits each containing one heavy (H) and one light (L) chain. The differences between antibody classes derive from their different H chains. H chains have a molecular weight of about 53 kDa, while L chains are about 23 kDa in mass.

Samsung Bioepis Exhibit No. 1002
Page 8599

Every individual "native" antibody has one type of L chain and one type of H chain, which are held together by disulfide bonds to form a heterodimeric subunit. Typically a native antibody (e.g., an IgG) has two such subunits, which are also held together by disulfide bonds. Within each chain, units of about 110 amino acid residues fold so as to form compact domains. Each domain is held together by a single intrachain disulfide bond. L chains have two domains, while H chains have four or five. Most H chains have a hinge region after the first (i.e., most amino-terminally located) two domains. The disulfide bonds linking together the heterodimeric subunits are located at the hinge regions. The hinge region is particularly sensitive to proteolytic cleavage, such proteolysis yielding two or three fragments (depending on the precise site of cleavage), a non-antigen binding fragment containing only H chain C regions (Fc) and one bivalent (Fab'2) or two monovalent (Fab) antigen binding fragments. The hinge region allows the antigen binding regions (each made up of a light chain and the first two domains of a heavy chain) to move freely relative to the rest of the native antibody, which includes the remaining heavy chain domains.

The first domain of each chain is highly variable in amino acid sequence, providing the vast spectrum of antibody binding specificities found in each individual. These are known as variable heavy (VH) and variable light (VL) domains. The second and subsequent (if any) domains of each chain are relatively invariant in amino acid sequence. These are known as constant heavy (CH) and constant light (CL) domains.

Each variable region contains three loops of hypervariable sequence that provide a complementary structure to that of the antigen and are critical in determining the antigen binding specificity of the antibody, as they are the contact sites for binding to the antigen. These loops are known as complementarity determining regions, or CDRs. Each variable domain is made up of three CDRs embedded in four much less variable framework segments (FRs). Together, the sets of collinear CDRs and FRs are in large part responsible for determining the three dimensional conformation of the variable

Samsung Bioepis Exhibit No. 1002
Page 8600

regions of antibody molecules.

CDRs and FRs are features that have been deduced from structural properties of antibody variable regions. Both amino acid sequence (primary structure) and three dimensional modeling (deduced secondary and tertiary structure) of antibody variable regions have been used by various researchers to define CDRs and, by default, FRs. While the positions of the CDRs are beyond question, not all workers in the art agree upon the precise locations of the boundaries of each CDR in VH or VL regions; there is no clear cut structural marker delineating CDR/FR boundaries.

Two definitions of CDR location are currently in general use in the art. These are the "sequence variability" definition of Kabat et al. ("Sequences of Proteins of Immunological Interest," 4th ed. Washington, D.C.: Public Health Service, N.I.H.) and the "structural variability" definition of Chothia and Lesk (J. Mol. Biol. 1987, 196:901). As used herein, the terms VL CDR1, VL CDR2, VL CDR3, VH CDR1, VH CDR2, and VH CDR3 refer minimally to the region of overlap between the regions designated for each CDR by each of these two definitions, and maximally to the total region spanned by the combination of the regions designated for each CDR by each of these two definitions.

One problem that antibody engineering attempts to address is the immune activity of a human patient that occurs in response to a native murine (or other non-human animal) antibody, typically a mAb, that is being administered to the patient for therapeutic purposes. This activity against murine antibodies is characterized by a human anti-mouse antibody (HAMA) response that can have deleterious effects on treatment efficacy and patient health. It has been found that almost all such human anti-non-human antibody ("HAMA type") activity is directed at the constant domains and at the FR regions of the variable domains of native non-human antibodies.

By manipulating the nucleic acid molecules encoding antibody H and L chains it is possible to incorporate non-human variable regions into antibodies otherwise made up of

Samsung Bioepis Exhibit No. 1002
Page 8601

human constant regions. The resulting antibodies are referred to as "chimeric antibodies," and are typically less prone to eliciting HAMA type responses than are the non-human antibodies from which the variable regions are derived.

An even more effective approach to eliminating the potential of a non-human antibody to elicit a HAMA type response is to "humanize" it, i.e., to replace its non-human framework regions with human ones. One way of achieving such humanization involves the insertion of polynucleotide fragments encoding the non-human CDRs of the antibody to be humanized into a nucleic acid molecule encoding an otherwise human antibody (with human constant regions if desired) so as to replace the human CDRs and to use the resulting nucleic acid molecule to express the encoded "humanized" antibody.

Unfortunately, however, humanization of non-human antibodies has unpredictable effects on antibody antigen interactions, e.g., antigen binding properties. Some of this unpredictability stems from the properties of the CDRs. Certain CDRs may be more amenable to the construction of humanized antibodies that retain the properties of the non-human CDR donor antibody than others. While the CDRs are key to the antigen binding properties of an antibody, CDRs and FRs must interact appropriately if the antigen specificity of an antibody is to be retained following humanization. The effects of combination with particular human FRs on uncharacterized non-human CDRs cannot be reliably predicted by any known method. However, the successful humanization of an antibody provides information that, in general, facilitates the successful humanization of the CDRs of that antibody using other human or altered human FRs. In addition, approaches are available that facilitate tailoring human FRs to enhance the likelihood of successful humanization.

Other problems addressed by antibody engineering include efficient antibody production and alteration of antibody pharmacokinetics. Recombinant protein production is generally most efficiently carried out in bacterial hosts. The large size and multimeric nature of native antibodies makes their production in bacteria difficult. One approach to dealing

Samsung Bioepis Exhibit No. 1002
Page 8602

with production problems is to use recombinant DNA methods to construct antibodies that have their H and L chains joined by a linker peptide to form a single chain (sc) antibody. As described below, there are several types of sc antibodies that can be constructed.

As is the case for humanization, the effects on antigen binding properties of constructing a particular type of sc antibody using H and L chains that have not been characterized with regard to their ability to function as part of an sc antibody cannot be reliably predicted by any known method. However, the successful construction of any one type of sc antibody from a particular native antibody provides information that, in general, facilitates the successful construction of other types of sc antibodies from that native antibody.

Single chain antibodies may include one each of only VH and VL domains, in which case they are referred to as scFv antibodies; they may include only one each of VH, VL, CH, and CL domains, in which case they are referred to as scFab antibodies; or they may contain all of the variable and constant regions of a native antibody, in which case they are referred to as full length sc antibodies.

The differing sizes of these antibodies imparts each with differing pharmacokinetic properties. In general, smaller proteins are cleared from the circulation more rapidly than larger proteins of the same general composition. Thus, full length sc antibodies and native antibodies generally have the longest duration of action, scFab antibodies have shorter durations of action, and scFv antibodies have even shorter durations of action. Of course, depending upon the illness being treated, longer or shorter acting therapeutic agents may be desired. For example, therapeutic agents for use in the prevention of immune and hemostatic disorders associated with extracorporeal circulation procedures (which are typically of brief duration) are preferably relatively short acting, while antibodies for the treatment of long term chronic conditions (such as inflammatory joint disease or GN) are preferably relatively long acting.

Samsung Bioepis Exhibit No. 1002
Page 8603

Detailed discussions of antibody engineering may be found in numerous recent publications including: Borrebaek, "Antibody Engineering, A Practical Guide," 1992, W.H. Freeman and Co. NY; and Borrebaek, "Antibody Engineering," 2nd ed. 1995, Oxford University Press, NY, Oxford.

SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of the present invention to provide a new approach for reducing the glomerular inflammation and kidney dysfunction associated with GN.

The method of the invention involves the use of preparations containing antibodies to human complement component C5 as pharmaceutical agents. More particularly, the invention provides for the use of anti-C5 antibodies that bind to complement component C5 or active fragments thereof. Preferably, the antibodies block the generation and/or activity of complement components C5a and C5b. For most applications, the antibody is a monoclonal antibody.

In the preferred embodiments of the invention, the administration of the anti-C5 antibody preparation is started after the appearance of GN symptoms, e.g., after the appearance of proteinuria. Alternatively, the invention can be used prophylactically to treat patients who are at risk for an acute exacerbation of existing GN, e.g., patients experiencing a flare-up of symptoms of systemic lupus erythematosus or similar autoimmune diseases that have resulted in GN.

As shown in the examples presented below, anti-C5 antibodies administered subsequent to the onset of GN essentially eliminate glomerular inflammation/enlargement and reduce kidney dysfunction (see Examples 1 and 2).

Although not wishing to be bound by any particular theory of operation, it is believed that the anti-C5 antibodies have these and other therapeutic effects through their activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9

Samsung Bioepis Exhibit No. 1002
Page 8604

membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The invention additionally provides compositions comprising anti-C5 antibodies that block complement hemolytic activity and C5a generation. These antibodies are useful for the treatment of GN as well as a number of other conditions. These include treatment of immune and hemostatic dysfunctions associated with extracorporeal circulation, treatment of inflammatory joint diseases, and other complement associated conditions, particularly inflammatory diseases.

Although other antibodies can be used to treat GN in accordance with the present invention, the novel antibodies of the invention are preferred. Preferably, these novel antibodies bind to the alpha chain of C5, but do not exhibit substantial binding to the alpha chain cleavage product C5a (referred to hereinafter and in the claims as "free C5a"). Other preferred targets for antibody binding include fragments of the alpha chain of human C5 that are immunoreactive with the most preferred antibody of the invention, the 5G1.1 antibody discussed below. Such preferred targets include the 46 kDa acid hydrolysis fragment of C5 (the "5G46k" fragment), the 27 kDa tryptic digestion fragment of C5 (the "5G27k" fragment), the 325aa peptide spanning amino acid residues 725-1049 of SEQ ID NO:2 (the "5G325aa" peptide), the 200 amino acid peptide spanning amino acids residues 850 to 1049 of SEQ ID NO:2 (the "5G200aa" peptide) - - as discussed below in Example 13.

The novel antibodies of the invention include antibodies that bind to an epitope within the amino acid sequence Val Ile Asp His Gln Gly Thr Lys Ser Ser Lys Cys Val Arg Gln Lys Val

Samsung Bioepis Exhibit No. 1002
Page 8605

Glu Gly Ser Ser, (SEQ ID NO:1) hereinafter referred to as the KSSKC epitope. These novel antibodies that bind to the KSSKC epitope are hereinafter referred to as anti-KSSKC antibodies, and monoclonal antibodies binding to the KSSKC epitope are hereinafter referred to as anti-KSSKC mAbs.

The novel antibodies of the invention have many advantages over other anti-C5 antibodies, particularly with regard for their use as anti-inflammatory therapeutic agents. These include the ability to substantially block both complement hemolytic activity and the generation of the proinflammatory complement cleavage product C5a to substantially the same extent at the same concentration of antibody. Some of the preferred antibodies of the invention have the additional advantageous property of blocking the binding of C5 to C3 or C4.

Particularly preferred antibodies of the invention are monospecific native anti-KSSKC antibodies. The 5G1.1 native anti-KSSKC mAb has the distinct advantage of substantially blocking both complement hemolytic activity and the generation of C5a at a stoichiometric ratio of antibody to C5 that approaches the theoretical one to two (antibody to antigen) limit of binding that can be achieved by a bivalent antibody. This is a desirable property because it allows smaller doses of antibody to achieve therapeutic effects than would be required of otherwise similar antibodies that cannot function at such a ratio.

The invention further provides recombinant mAbs that are derivatives (including monovalent derivatives) of these native mAbs. These include anti-KSSKC recombinant mAbs. Preferably the antibodies of the invention provide a level of blockade of both complement hemolytic activity and C5a generation (on a per mole of binding site basis) that is obtained when the antibody concentration is within an order of magnitude of that of the native mAbs. Particularly preferred anti-KSSKC recombinant mAbs provide a level of such blockade when the antibody concentration is no more than three fold that of the native mAbs of the invention.

The invention further provides nucleic acid sequences of

Samsung Bioepis Exhibit No. 1002
Page 8606

polynucleotides encoding such recombinant anti-KSSKC mAbs, as well as amino acid sequences of the polypeptides encoded by these nucleic acid molecules of the invention.

The invention further provides CDR sequences that are useful in the construction of the humanized antibodies of the invention, as well as peptides and oligopeptides that are useful in the preparation and characterization of the antibodies of the invention.

Anti-C5 antibodies of the invention have activity in blocking the generation or activity of the C5a and/or C5b active fragments of complement component C5. Through this blocking effect, the antibodies inhibit the proinflammatory (anaphylatoxic) effects of C5a and the generation of the C5b-9 membrane attack complex (MAC). Significantly, the blockage effected by the anti-C5 antibodies, since it occurs at the level of complement component C5, has the advantage of maintaining important opsonic, anti-infective, and immune complex clearance functions of the complement system mediated by, inter alia, complement component C3.

The accompanying figures, which are incorporated in and constitute part of the specification, illustrate certain aspects of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the figures and the description are explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A, 1B, and 1C -- Photomicrographs of PAS stained sections of mouse kidneys. Fig 1A -- uninduced untreated mouse. Fig. 1B -- GN-induced PBS-(control)-treated mouse. Fig. 1C -- GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400X.

Figures 2A, 2B, and 2C -- Photomicrographs of immunofluorescence stained sections of mouse kidneys. Fig 2A- - uninduced untreated mouse. Fig. 2B -- GN-induced PBS-(control)-treated mouse. Fig. 2C -- GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 200X.

Samsung Bioepis Exhibit No. 1002
Page 8607

Figure 3 -- Results of hemolytic (cell lysis) assays of serum from GN-induced animals treated with either anti-C5 antibodies in PBS ("Anti-C5") or PBS alone ("PBS control"). Also shown are the results of assays performed with normal serum.

Figure 4 -- Results of soluble C5b-9 ("sC5b-9") assays. "ND" indicates not determined.

Figures 5A, 5B, and 5C -- Immunofluorescence photomicrographs of kidney sections stained for mouse C3. Fig 5A -- uninduced untreated mouse. Fig. 5B -- GN-induced PBS-(control)-treated mouse. Fig. 5C -- GN-induced anti-C5 treated mouse. Magnification for each is the same, approximately 400X.

Figure 6 -- Results of C3a assays of samples of circulating human blood. "ND" indicates not determined.

Figures 7A and 7B -- Pharmacokinetic analyses of the reduction of the cell lysis ability of mouse (Fig. 7A) or human (Fig. 7B) blood after treatment with anti-C5 antibodies.

The immunofluorescent staining of Figures 2 and 5 is confined to the glomerular capillary network (tuft) and thus the enlargement of the glomerulus seen in Figure 1B is not visible in Figures 2B and 5B.

Figure 8 -- Scatchard analysis of native 5G1.1 binding to C5.

Figure 9 -- Scatchard analysis of native N19/8 binding to C5.

Figure 10 -- C3a generation in samples of circulating human blood in the presence of native 5G1.1.

Figure 11 -- sC5b-9 generation in samples of circulating human blood in the presence of native 5G1.1.

Figure 12 -- Serum hemolytic activity of samples of circulating human blood in the presence of native 5G1.1.

Figure 13 -- Serum hemolytic activity in the presence of m5G1.1 scFv.

Figure 14 -- C5a generation in the presence of m5G1.1 scFv.

Figure 15 -- C3a generation in samples of circulating human blood in the presence of m5G1.1 scFv.

Figure 16 -- Serum hemolytic activity of samples of circulating human blood in the presence of 5G1.1 scFv.

Figure 17 -- sC5b-9 generation in samples of circulating human blood in the presence of m5G1.1 scFv.

Figure 18 -- The light chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are number according to Kabat et al., *supra*. Boxed amino acids correspond to peptide sequences obtained from the mature 5G1.1 light chain or from an endoproteinase Lys C peptide of 5G1.1. The complementarity determining region (CDR) residues according to the sequence variability definition and the structural variability definition are underlined and overlined, respectively.

Figure 19 -- The heavy chain variable region of the antibody 5G1.1. Sequence derived from the 5' oligonucleotide primer used for PCR amplification of the variable region is shown in lower case. Amino acids are numbered using the scheme of Kabat et al. *supra* with +1 denoting the first amino acid of the processed mature variable region. Boxed amino acids correspond to peptide sequence obtained from the 5G1.1 heavy chain after treatment with pyroglutamate aminopeptidase. The complementarity determining region (CDR) residues according to the sequence variability definition or according to the structural variability definition are underlined and overlined, respectively.

BACKGROUND PHYSIOLOGY & PATHOLOGY

The discussion in this section is not limited to subject matter that qualifies as "prior art" against the present invention. Therefore, no admission of such prior art status shall be implied or inferred by reason of inclusion of particular subject matter in this discussion, and no declaration against the present inventors' interests shall be implied by reason of such inclusion.

I. Introduction

As described above, the present invention relates to therapeutic treatments for GN and other immune complex

Samsung Bioepis Exhibit No. 1002
Page 8609

mediated diseases, as well as to the treatment of other complement mediated diseases and to the inhibition of complement component C5. To provide background for the description of the preferred embodiments and the examples presented below, we turn first to general discussions of the complement arm of the immune system, the pathophysiologic features of GN, and previous studies of the role of complement in GN pathogenesis.

General discussions of the complement system and GN can be found in, for example, Glassock and Brenner, 1994; Couser, 1993; Couser, 1992; Couser, et al, 1992; Rich, 1992; Glassock and Brenner, 1987; Robbins and Cotran, 1979; and Guyton, 1971.

II. The Complement System

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and while they differ in their initial steps, they converge and share the same "terminal complement" components (C5 through C9) responsible for the activation and destruction of target cells.

The classical complement pathway is typically initiated by antibody recognition of and binding to an antigenic site on a target cell. The alternative pathway is usually antibody independent, and can be initiated by certain molecules on pathogen surfaces. Both pathways converge at the point where complement component C3 is cleaved by an active protease (which is different in each pathway) to yield C3a and C3b. Other pathways activating complement attack can act later in

Samsung Bioepis Exhibit No. 1002
Page 8610

the sequence of events leading to various aspects of complement function.

C3a is an anaphylatoxin (see discussion below). C3b binds to bacterial and other cells, as well as to certain viruses and immune complexes, and tags them for removal from the circulation. (C3b in this role is known as opsonin.) The opsonic function of C3b is considered to be the most important anti-infective action of the complement system. Patients with genetic lesions that block C3b function are prone to infection by a broad variety of pathogenic organisms, while patients with lesions later in the complement cascade sequence, i.e., patients with lesions that block C5 functions, are found to be more prone only to Neisseria infection, and then only somewhat more prone (Fearon, in Intensive Review of Internal Medicine, 2nd Ed. Fanta and Minaker, eds. Brigham and Women's and Beth Israel Hospitals, 1983).

C3b also forms a complex with other components unique to each pathway to form classical or alternative C5 convertase, which cleaves C5 into C5a and C5b. C3 is thus regarded as the central protein in the complement reaction sequence since it is essential to both the alternative and classical pathways (Wurzner, et al., Complement Inflamm. 8:328-340, 1991). This property of C3b is regulated by the serum protease Factor I, which acts on C3b to produce iC3b. While still functional as opsonin, iC3b cannot form an active C5 convertase.

C5 is a 190 kDa beta globulin found in normal serum at approximately 75μg/ml (0.4μM). C5 is glycosylated, with about 1.5-3 percent of its mass attributed to carbohydrate. Mature C5 is a heterodimer of a 999 amino acid 115 kDa alpha chain that is disulfide linked to a 656 amino acid 75 kDa beta chain. C5 is synthesized as a single chain precursor protein product of a single copy gene (Haviland et al. J. Immunol. 1991, 146:362-368). The cDNA sequence of the transcript of this gene predicts a secreted pro-C5 precursor of 1659 amino acids along with an 18 amino acid leader sequence (SEQ ID NO:2).

The pro-C5 precursor is cleaved after amino acid 655 and 659, to yield the beta chain as an amino terminal fragment

Samsung Bioepis Exhibit No. 1002
Page 8611

(amino acid residues 1 to 655 of SEQ ID NO:2) and the alpha chain as a carboxyl terminal fragment (amino acid residues 660 to 1658 of SEQ ID NO:2), with four amino acids (amino acid residues 656-659 of SEQ ID NO:2) deleted between the two.

C5a is cleaved from the alpha chain of C5 by either alternative or classical C5 convertase as an amino terminal fragment comprising the first 74 amino acids of the alpha chain (i.e., amino acid residues 660-733 of SEQ ID NO:2). Approximately 20 percent of the 11 kDa mass of C5a is attributed to carbohydrate. The cleavage site for convertase action is at or immediately adjacent to amino acid residue 733 of SEQ ID NO:2. A compound that would bind at or adjacent to this cleavage site would have the potential to block access of the C5 convertase enzymes to the cleavage site and thereby act as a complement inhibitor.

C5 can also be activated by means other than C5 convertase activity. Limited trypsin digestion (Minta and Man, J. Immunol. 1977, 119:1597-1602; Wetsel and Kolb, J. Immunol. 1982, 128:2209-2216) and acid treatment (Yammamoto and Gewurz, J. Immunol. 1978, 120:2008; Damerau et al., Molec. Immunol. 1989, 26:1133-1142) can also cleave C5 and produce active C5b.

C5a is another anaphylatoxin (see discussion below). C5b combines with C6, C7, and C8 to form the C5b-8 complex at the surface of the target cell. Upon binding of several C9 molecules, the membrane attack complex (MAC, C5b-9, terminal complement complex -- TCC) is formed. When sufficient numbers of MACs insert into target cell membranes the openings they create (MAC pores) mediate rapid osmotic lysis of the target cells. Lower, non-lytic concentrations of MACs can produce other effects. In particular, membrane insertion of small numbers of the C5b-9 complexes into endothelial cells and platelets can cause deleterious cell activation. In some cases activation may precede cell lysis.

As mentioned above, C3a and C5a are anaphylatoxins. These activated complement components can trigger mast cell degranulation, which releases histamine and other mediators of inflammation, resulting in smooth muscle contraction, increased vascular permeability, leukocyte activation, and

Samsung Bioepis Exhibit No. 1002
Page 8612

other inflammatory phenomena including cellular proliferation resulting in hypercellularity. C5a also functions as a chemotactic peptide that serves to attract pro-inflammatory granulocytes to the site of complement activation.

III. Pathophysiology of GN

Although GN may accompany an extraordinary range of pathologic processes, in general it is encountered most commonly in the course of infectious diseases, in autoimmunity, and as a consequence of therapy for some other disease process. The causative mechanism for GN is typically the deposit of circulating immune complexes in the kidney. Factors involved in the pathogenesis of GN include the specific antigen and antibody involved and the inflammatory processes that occur as a consequence of immune complex deposition.

Antigens Involved in the Formation of Immune Complexes That Cause GN: Antigens involved in the development of GN can be broadly classified as endogenous, infectious, and iatrogenic (those encountered as a consequence of medical practice). In many cases the specific antigen is unknown, although the general class can usually be identified.

The best known example of the formation of endogenous immune complexes is the DNA anti-DNA complexes produced in connection with systemic lupus erythematosus (lupus, SLE). Other important sources of endogenous antigens include malignancies in which immune complex formation may contribute to the development of paraneoplastic syndromes.

Infections with organisms of many types, particularly chronic infections, are also associated with the development of immune complexes that can cause GN. Bacterial and fungal infections that can produce such complexes include infection with certain strains of streptococci, Pseudomonas, disseminated gonococcal infection, lepromatous leprosy, subacute bacterial endocarditis, bronchopulmonary aspergillosis, secondary syphilis, and chronic infections in patients with cystic fibrosis.

Viral diseases in which immune complex deposition may be a prominent feature include hepatitis B infection, dengue,

Samsung Bioepis Exhibit No. 1002
Page 8613

infectious mononucleosis, and subacute sclerosing panencephalitis. GN is also a prominent feature of many parasitic infestations such as the GN seen in children with quartan malaria, as well as toxoplasmosis, trypanosomiasis, and schistosomiasis.

Iatrogenic antigens constitute a special class of exogenous antigens. These include those responsible for the prototype immune complex disease, serum sickness, which follows formation of immune complexes between heterologous serum constituents and autologous antibodies. Serum sickness was regularly seen earlier in this century when infectious diseases were frequently treated with heterologous antisera.

An iatrogenic disease essentially indistinguishable from classic serum sickness can occur as a consequence of high-dose antibiotic therapy. The serum sickness-like manifestations of immune responses to these drugs include GN and reflect the fact that certain drugs, particularly the ß-lactam and sulfonamide antibiotics, are effective haptens that are capable of inducing antibody responses upon spontaneous conjugation to autologous proteins.

Factors Affecting Immune Complex Formation and Deposition: Features of both antigen and antibody determine the likelihood of pathologic immune complex formation and subsequent deposition in the kidney. Chief among these are the absolute concentrations of the reactants and their relative molar ratios.

Most antigens display multiple epitopes and typically stimulate a polyclonal antibody response. All naturally occurring antibody molecules are at least bivalent. These properties allow for the formation of an extensive antigen-antibody lattice, the size of which is determined largely by the affinity of the antibodies and the molar ratio of antigen to antibody.

In general, antibody responses begin under conditions in which antigen is present in excess to antibody, and this relative ratio changes as the antibody response increases in magnitude. Complexes formed initially are usually small and exhibit little or no pathogenic activity. In contrast, very

Samsung Bioepis Exhibit No. 1002
Page 8614

large complexes are often formed as the amount of antigen becomes limiting, late in the course of an antibody response under conditions of antibody excess. Because these very large complexes are readily cleared by the reticuloendothelial system in the liver, they are also relatively nonpathogenic.

The formation of immune complexes that can cause GN is believed to occur during conditions of slight antigen excess or near the point of antibody-antigen equivalence, where lattice formation is maximal and lattice size is large, but not very large.

Several factors influence the speed and location of immune complex precipitation. Interactions between Fc regions of antibody molecules promote rapid precipitation of immune complexes. The role of Fc-Ec interactions in immune complex precipitation is illustrated by studies of the properties of F(ab')2 antibody fragments, which do not contain Fc regions. Although the valence of F(ab')2 fragments does not differ from that of most whole immunoglobulins, F(ab')2 antibody fragments form lattices more slowly.

Antigen charge plays a role in determining the tissue localization of sites of deposition of immune complex precipitates. Complexes with a substantial positive charge are preferentially attracted to the strong negative charge of basement membranes, particularly in the renal glomerulus.

Localized presence of antigen may largely account for certain cases of organ specific immune complex deposition. Diseases such as Goodpasture's syndrome (a rare form of GN) are typically not classified as immune complex diseases because the complexes are formed in situ in the kidney rather than being preformed in the circulation and then deposited. Once the immune complexes are formed, the subsequent inflammatory process is believed to be essentially the same as that seen following deposition of preformed complexes. However, the different mode of deposition distinguishes this syndrome from typical GN caused by circulating immune complexes.

Features of blood flow and vascular structure are also important in determining the localization of immune complex

Samsung Bioepis Exhibit No. 1002
Page 8615

deposits. Chief among these is capillary permeability. Because their capillary endothelium is fenestrated, renal glomeruli are preferential sites for the deposition of immune complexes. Hemodynamic variables enhancing immune complex localization include turbulence of flow and increased blood pressure, both of which are present in the renal glomeruli.

Complement and Complement Receptors as Regulators of Immune Complex Deposition: In addition to their proinflammatory functions, complement components can also inhibit immune complex deposition and resolubilize immune complex precipitates from sites of deposition. In addition, it is known that erythrocyte receptors for C3b, e.g., CR1, are important for reticuloendothelial clearance of opsonized circulating immune complexes.

Analysis of the clinical pattern of immune complex disease in patients with deficiencies of particular complement components provides information regarding the normal role of these components in the prevention of complex deposition. The incidence of immune complex disease in patients with deficiencies of Clq, Clr, Cls, C4, C2, or C3 varies from 60 to 90 percent, with the majority of these patients exhibiting a lupus-like syndrome. Immune complex disease is rarely associated with deficiencies of late-acting or alternative pathway components.

The binding of complement components to immune complexes prevents the formation of large antigen-antibody lattices and inhibits immune precipitation. This process requires activation via the classical pathway; serum that is deficient for Clq, C4, or C2 does not effectively inhibit lattice formation and complex precipitation. Classical pathway dependence may reflect the initial binding of C1 components, impeding the Fc-Fc interactions between IgG molecules that contribute to immune precipitation. This is followed by covalent binding of C3b to the complexes, which further inhibits immune precipitation and leads to solubilization of previously deposited complexes.

The solubilization process also depends upon activation of components of the alternative pathway. Consequently, by

Samsung Bioepis Exhibit No. 1002
Page 8616

promoting clearance of immune complexes and inhibiting their deposition at sites of inflammation, complement components and their receptors serve as negative regulators of immune complex diseases that may retard disease development.

It should be noted that the present invention involves blocking the activities of complement component C5. The targeting of this component does not alter the functions of the early complement components, and thus does not compromise the negative regulatory effects on immune complex deposition of those early components.

Immune Complex-Mediated Inflammation: Basophils are important in the initiation of immune complex-mediated inflammatory responses, as capillary permeability is markedly increased by the action of vasoactive amines such as histamine and platelet-activating factor, which are released by these cells. Vascular permeability is also promoted by aggregation of platelets at sites of an inflammatory lesion, with the release of platelet-activating factor and the formation of microthrombi.

Basophil degranulation may reflect the effects of IgE antibodies, as well as the elaboration of the anaphylatoxin components of complement, C3a and C5a.

In addition to basophils and platelets, the primary cellular effectors of immune complex-mediated inflammation are polymorphonuclear leukocytes, monocytes, and macrophages.

IV. Previous Studies of the Role of Complement in GN Pathogenesis

Extensive work has been performed in an attempt to understand the possible role of complement in the development of GN. This work has included studies of GN using a number of animal models by, among others, Unanue, et al., (1964); Cochrane, et al., (1965); Kniker, et al., (1965); Salant, et al., (1980); Groggel, et al., (1983); Falk and Jennette (1986); Jennette, et al., (1987); Passwell, et al., (1988); Schrijver, et al., (1988); Baker, et al., (1989); Schrijver, et al., (1990); Couser, et al., (1991); and Couser, et al., (1992).

Samsung Bioepis Exhibit No. 1002
Page 8617

These studies have shown that complement plays a role in GN pathogenesis. However, they have not established specific unequivocal roles for the various complement components. In particular, the relative roles of C3 and other anaphylatoxins compared to the roles of the terminal complement components in GN pathogenesis have not been unequivocally established. Also, some researchers have reported that complement depletion does not diminish glomerular injury. See Kniker, et al., (1965).

The foregoing work includes that of Falk and Jennette (1986), who reported results of experiments in which attempts were made to induce GN in mice having a genetic defect that resulted in a deficiency of complement component C5. The report concludes that C5 or some terminal complement component dependent on C5 plays a role in the pathogenesis of GN.

Significantly, with regard to the present invention, Falk and Jennette in no way disclose or suggest that an antibody to C5 can be used to treat GN. Indeed, it would be counterintuitive to use an antibody to treat disease which typically involves the formation and deposition of circulating antibody-antigen immune complexes. Plainly, the creation of more circulating immune complexes would seem to be the last way to go to solve a problem that can be caused by circulating immune complexes. Yet, as demonstrated by the surprising results presented below, anti-C5 antibodies have been found to effectively block GN, even though the creation of additional circulating immune complexes is inherent in their mode of action.

Baker et al. (1989), Couser et al. (1991), and Couser et al. (1992) (hereinafter referred to collectively as the "C6" work) discuss experiments in which high levels of an anti-C6 polyclonal antibody preparation were administered to rats, following which immune complexes were formed in situ in the rats' kidneys. Significantly, with regard to the present invention, the anti-C6 antibody preparation was not administered to animals with pre-existing kidney disease, i.e., it was not used as a therapeutic treatment. Moreover, the experimental protocol used in the C6 experiments did not

Samsung Bioepis Exhibit No. 1002
Page 8618

involve circulating immune complexes, but rather involved complexes formed in situ. Accordingly, the experiments did not disclose or suggest the counterintuitive approach of the present invention wherein more circulating immune complexes are formed in the process of treating a disease state caused by circulating immune complexes.

Further, the anti-C6 antibody dosages used in the C6 work were too high for practical medical use. Specifically, these antibodies were used at a dosage of 1 gm/kg, a dosage which would correspond to 70 gm of antibody for a 70 kg (155 lb) individual. In contrast, the anti-C5 antibodies used in the practice of the present invention are used at concentrations at or below 0.1 gm/kg, i.e., a factor of at least ten times less than used in the C6 work. Indeed, as shown by the examples presented below, anti-C5 antibody dosages as low as 0.03 gm/kg, i.e., 33 times less than those used in the C6 work, have been found to achieve the therapeutic effects of the invention in treating GN. For a 70 kg individual, this antibody level corresponds to a dose of just 2.1 gms.

The novel anti-KSSKC antibodies of the invention allow the use of even lower dosage levels to treat GN and other inflammatory conditions. Based upon their level of activity in human blood, they are expected to provide complete complement inhibition at dosages below 0.005g/kg, and to provide therapeutically effective complement inhibition at dosages below 0.003g/kg. This 3mg/kg dosage is one tenth the dosage discussed below in Examples 4 and 5 for the for the anti-C5 (beta chain specific) mAb N19/8. Some of the full length anti-KSSKC mabs of the invention will provide therapeutic benefits even at dosages below 0.0022g/kg. This is the minimum dose providing complete complement inhibition as calculated from the data obtained using the anti-KSSKC 5G1.1 mAb in human blood in a CPB circuit, as discussed below in Example 9.

Accordingly, dosages of less than 0.005g/kg are preferred, with dosages of below 0.003g/kg being more preferred, and dosages below 0.0022g/kg being particularly preferred. For a 70 kg individual, these antibody dosage levels correspond to a

Samsung Bioepis Exhibit No. 1002
Page 8619

dose of less than 0.35 gms for the highest dosage of the preferred dosages, less than 0.21 gms for the more preferred dosage, and less than or equal to 0.15 gms for the most preferred dosage.

Of course, dosage levels of single chain and other recombinant mAbs of the invention must be adjusted according to their level of activity (e.g., their binding affinity, their ability to block C5 activation, and/or their ability to block complement hemolytic activity), their valency, and their molecular weight. For example, the humanized scFv anti-KSSKC mAbs of Example 11 are approximately 27 kDa, about one sixth the approximately 155 kDa mass of a native, full length IgG antibody. These antibodies completely block complement hemolytic activity and C5a generation at a ratio of 3:1, six fold greater than for native 5G1.1 (but only three fold greater when viewed in terms of numbers of antibody-antigen binding sites).

Thus, the number of molecules of each of these scFvs required to equal the effect of a single molecule of native 5G1.1 must be increased by a factor of six to adjust for the ratio at which blocking is complete. Since the mass of these molecules is approximately one sixth of the mass of native 5G1.1, dosages of the scFvs are in the same range as those for the native 5G1.1 mAb.

In addition to lowering dosage levels, the anti-C5 antibodies used in the practice of the present invention (i.e., in treating GN) achieve important therapeutic effects not achieved with the anti-C6 antibodies. Specifically, the control and test animals in the C6 work exhibited both hypercellularity and narrowing of capillary lumens. In direct contrast, no such hypercellularity or narrowing of capillary lumens was seen when diseased individuals were treated with anti-C5 antibodies (see Figure 1).

Moreover, the anti-C5 antibodies used in the present invention achieve a reduction in glomerular enlargement, thus providing a clear demonstration of the unexpectedly powerful anti-inflammatory effects of the anti-C5 antibodies used in the practice of the invention. Nowhere in the C6 work is

Samsung Bioepis Exhibit No. 1002
Page 8620

there any disclosure or suggestion of such a powerful anti-inflammatory effect.

V. Anti-C5 Monoclonal Antibodies That Block Complement Hemolytic Activity and Block the Generation of C5a:

Anti-C5 mAbs that have the desirable ability to block complement hemolytic activity and to block the generation of C5a (and are thus preferred for use in the treatment of GN and other inflammatory conditions in accordance with the present invention) have been known in the art since at least 1982 (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323). Antibodies known in the art that are immunoreactive against C5 or C5 fragments include antibodies against the C5 beta chain (Moongkarndi et al. Immunobiol. 1982, 162:397; Moongkarndi et al. Immunobiol. 1983, 165:323; Wurzner et al. 1991, *supra*; Mollnes et al. Scand. J. Immunol. 1988, 28:307-312); C5a (see for example, Ames et al. J. Immunol. 1994, 152:4572-4581, U.S. patent No. 4,686,100, and European patent publication No. 0 411 306); and antibodies against non-human C5 (see for example, Giclas et al. J. Immunol. Meth. 1987, 105:201-209). Significantly, none of these anti-C5 mAbs has the properties of the novel anti-C5 mAbs of the invention, i.e., none of them binds to the C5 alpha chain but not to the C5 cleavage product C5a, none of them has the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody. It is noteworthy that an scFv derivative of the N19/8 antibody of Wurzner et al. 1991, *supra*, has been prepared, and that the N19/8 scFv has 50% less inhibitory activity towards C5a generation than the native N19/8 antibody (see Example 15). This is in contrast to the 5G1.1 scFv, which retained substantially all of its inhibitory activity towards C5a generation (see Example 12).

While not wishing to be bound by any particular theory of operation, it is believed that these distinctions are due to the specific binding characteristics of the antibodies of the invention. Accordingly, antibodies that do not bind to sites within the alpha chain of C5, and antibodies that bind to the

Samsung Bioepis Exhibit No. 1002
Page 8621

C5 cleavage product C5a (free C5a), are believed to lack the ability to substantially block both complement hemolytic activity and the generation of C5a to substantially the same extent at the same concentration of antibody.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to the use of anti-C5 antibodies in treating patients suffering from GN and other diseases, and to specific C5 antibodies and antibody preparations. Preferably, and when used to treat GN, the anti-C5 antibodies are used in an amount effective to substantially reduce (e.g., reduce by at least about 50%) the cell-lysing ability of complement present in the patient's blood (the "cell-lysing ability of complement present in the patient's blood" is also referred to herein as the "serum complement activity of the patient's blood"). Reduction of the cell-lysing ability of complement present in the patient's blood can be measured by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays."

To achieve the desired reductions, the anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments will also require differing dosages than the equivalent intact immunoglobulins, as they are of considerably smaller mass than intact immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

The dose will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician. Dosage levels of the antibodies for human subjects are generally between about 1 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 5 mg per kg and about 50 mg per kg per patient per treatment. In terms of plasma concentrations, the antibody concentrations

Samsung Bioepis Exhibit No. 1002
Page 8622

are preferably in the range from about 25 µg/ml to about 500 µg/ml.

Subject to the judgement of the physician, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the monitoring of clinical endpoints such as BUN levels, proteinuria levels, etc., with the dosage levels adjusted as needed to achieve the desired clinical outcome. Alternatively, levels of serum complement activity available in the patient's blood are monitored using the techniques set forth below under the heading "Cell Lysis Assays" to determine if additional doses or higher or lower dosage levels of antibodies are needed, with such doses being administered as required to maintain at least about a 50% reduction, and preferably about a 95% or greater reduction of serum complement activity. Other protocols can, of course, be used if desired as determined by the physician.

Administration of the anti-C5 antibodies will generally be performed by an intravascular route, e.g., via intravenous infusion by injection. Other routes of administration may be used if desired. Formulations suitable for injection are found in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). Such formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline, buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. The formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

The formulations of the invention can be distributed as articles of manufacture comprising packaging material and the anti-C5 antibodies. When prepared for use in the treatment of GN, the packaging material will include a label which indicates that the formulation is for use in the treatment of kidney disease and may specifically refer to nephritis or glomerulonephritis.

Samsung Bioepis Exhibit No. 1002
Page 8623

The anti-C5 antibody is preferably a monoclonal antibody, although polyclonal antibodies produced and screened by conventional techniques can also be used if desired. As discussed above, the anti-C5 antibodies must be effective in reducing the cell-lysing ability of complement present in human blood. As also discussed above, this property of the antibodies can be determined by methods well known in the art such as, for example, by the chicken erythrocyte hemolysis method described below under the heading "Cell Lysis Assays".

The anti-C5 antibodies used in the practice of the invention bind to C5 or fragments thereof, e.g., C5a or C5b. Preferably, the anti-C5 antibodies are immunoreactive against epitopes on the beta chain of purified human complement component C5 and are capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. This capability can be measured using the techniques described in Wurzner, et al., Complement Inflamm 8:328-340, 1991. Preferably, the anti-C5 antibodies are used to treat GN in an amount effective to reduce the C5 convertase activity available in the patient's blood by at least about 50%.

In a particularly preferred embodiment of the invention, the anti-C5 antibodies are not immunoreactive against epitopes on the beta chain, but rather are immunoreactive against epitopes within the alpha chain of purified human complement component C5. In this embodiment the antibodies are also capable of blocking the conversion of C5 into C5a and C5b by C5 convertase. In an especially preferred example of this embodiment they can provide this blockade at substantially the same concentrations needed to block hemolytic activity.

Within the alpha chain, the most preferred antibodies bind to an amino-terminal region, however, they do not bind to free C5a. Particularly preferred targets for these antibodies within the alpha chain include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope. The scope of the invention also includes the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa peptide, or the KSSKC epitope that are useful as immunogens and screening ligands for producing the antibodies

Samsung Bioepis Exhibit No. 1002
Page 8624

of the invention.

Hybridomas producing monoclonal antibodies reactive with complement component C5 can be obtained according to the teachings of Sims, et al., U.S. Patent No. 5,135,916. As discussed therein, antibodies are prepared using purified components of the complement membrane attack complex as immunogens. In accordance with the present invention, complement component C5 or C5b is preferably used as the immunogen. In accordance with a particularly preferred aspect of the present invention, the immunogen is the alpha chain of C5. Within the alpha chain, the most preferred immunogens include the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, or the 5G200aa peptide. A less preferred immunogen is the KSSKC epitope.

In accordance with the invention, the antibodies of the invention all share certain required functional properties. These are the ability to substantially inhibit complement hemolytic activity and to substantially inhibit the conversion of C5 to produce C5a. Preferably, but not requisitely, they provide these functions when used at a molar ratio of antibody to antigen (C5) of 3:1 or less.

A particularly preferred antibody of the invention is the 5G1.1 antibody (5G1.1, produced by the 5G1.1 hybridoma, ATCC designation HB-11625, April 27, 1994). Other particularly preferred antibodies of the present invention share the required functional properties discussed in the preceding paragraph and have any of the following characteristics:

(1) they compete with 5G1.1 for binding to portions of C5 - - the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa" peptide or the KSSKC peptide - - that are specifically immunoreactive with 5G1.1; and

(2) they specifically bind to the C5 alpha chain, the 5G46k fragment, the 5G27k fragment, the 5G325aa peptide, the 5G200aa" peptide, and/or the KSSKC peptide. Such specific binding, and competition for binding can be determined by various methods well known in the art, including the plasmon surface resonance method (Johne et al., J. Immunol. Meth.

Samsung Bioepis Exhibit No. 1002
Page 8625

1993, 160:191-198).

(3) they block the binding of C5 to either C3 or C4 (which are components of C5 convertase).

Also in accordance with the invention, the antibodies preferably should prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. In a particularly preferred embodiment, these anti-C5 antibodies will not impair the opsonization function associated with the activation of complement component C3 by a C3 convertase. Plasma C3 convertase activity can be measured by assaying plasma for the presence of C3a as described below under the heading "Histology." Preferably, the anti-C5 antibody produces essentially no reduction in plasma C3a levels.

General methods for the immunization of animals (in this case with C5 or C5b or another preferred immunogen), isolation of polyclonal antibodies or antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate Hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case C5 or C5b or another preferred immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz, et al., Cellular Immunol. 127:337-351, 1990; Wurzner, et al., Complement Inflamm. 8:328-340, 1991; and Mollnes, et al., Scand. J. Immunol. 28:307-312, 1988.

As used herein, the term "antibodies" refers to immunoglobulins produced in vivo, as well as those produced in vitro by a hybridoma, and antigen binding fragments (e.g.,

Samsung Bioepis Exhibit No. 1002
Page 8626

Fab' preparations) of such immunoglobulins, as well as to recombinantly expressed antigen binding proteins, including immunoglobulins, chimeric immunoglobulins, "humanized" immunoglobulins, antigen binding fragments of such immunoglobulins, single chain antibodies, and other recombinant proteins containing antigen binding domains derived from immunoglobulins. As used herein, "antibodies" also refers to antigen binding synthetic peptides comprising sequences derived from the sequences of immunoglobulin antigen binding domains. As used herein, the term "recombinant mAbs" refers to recombinantly expressed antigen binding proteins. As used herein, the term "antibody-antigen binding site" refers to an antigen binding site of an antibody comprising at least one CDR sequence.

Antibodies whose amino acid sequences are full length immunoglobulin sequences that have not been truncated (e.g., to produce an scFv or an Fab) or mutated (e.g., spliced to form a chimeric antibody or humanized) are referred to herein as "native" antibodies. Publications describing methods for the preparation of such antibodies, in addition to those listed immediately above, include: Reichmann, et al., Nature, 332:323-327, 1988; Winter and Milstein, Nature, 349:293-299, 1991; Clackson, et al., Nature, 352:624-628, 1991; Morrison, Annu Rev Immunol, 10:239-265, 1992; Haber, Immunol Rev, 130:189-212, 1992; and Rodrigues, et al., J Immunol, 151:6954-6961, 1993.

While treatment of GN in accordance with the process of the present invention may be carried out using polyclonal or monoclonal antibodies, monospecific antibodies are preferred. As used herein "monospecific antibodies" refer to antibodies that bind to a specific region of a particular antigen. All monoclonal antibodies are monospecific, but polyclonal antibodies are typically not monospecific.

As is known in the art, however, monospecific polyclonal antibodies may be prepared by various methods. For example, a peptide (e.g., an oligopeptide --- as used hereinafter and in the claims, a polymer of 5 to 200 amino acids) may be used as an immunogen. Another procedure allowing the preparation of

Samsung Bioepis Exhibit No. 1002
Page 8627

monospecific polyclonal antibodies is the use of antigen affinity purification techniques to isolate a monospecific antibody population from a polyclonal antibody mixture. In accordance with the present invention, peptides are preferred as immunogens for the production and as affinity ligands for the purification of monospecific polyclonal anti-KSSKC antibodies.

The native (i.e., non-engineered) monoclonal antibodies of the invention are preferably prepared by conventional means, with the 5G46k fragment, the 5G27k fragment, the 5G200aa peptide, the 5G325aa peptide, and/or the KSSKC peptide (e.g., immobilized on a polypropylene membrane as described below in Example 13) being used as screening ligand(s). This involves testing hybridoma supernatants for binding to each screening ligand.

In one preferred embodiment, the native mAbs of the invention are prepared using the alpha chain of human C5, or fragments thereof, as immunogen. Preferred fragments of the alpha chain of human C5 for this purpose include the 5G46k fragment, the 5G27k fragment, and/or the 5G200aa fragment. Although less preferred, the KSSKC peptide may also be used as an immunogen.

Another (albeit less preferred) immunogen and screening ligand for the preparation of antibodies within the scope of the novel antibodies of the present invention is the "cleavage site peptide," i.e., the peptide spanning amino acids 725 through 754 of SEQ ID NO:2 (the C5a cleavage site), as discussed below in Example 13.

In another preferred embodiment of the invention, the native mAbs of the invention are prepared in transgenic mice expressing human immunoglobulins (see, for example, Green et al., Nature Genet. 1994, 7:13-21). In this case, the same preferred immunogens and screening ligands are used as described for the preparation of other native mAbs.

In another preferred embodiment of the invention, the recombinant mAbs of the invention are prepared by screening phage display libraries expressing recombinant mAb-encoding polynucleotides (preferably encoding human recombinant mAbs).

Samsung Bioepis Exhibit No. 1002
Page 8628

See, for example, Ames et al., 1994, *supra*; Smith and Scott, Meth. Enzymol. 1993, 217:228; Kay et al., Gene, 1993, 128:59-65. This screening is carried out with the screening ligands described above for the preparation of native mAbs. The recombinant mAbs of the invention are prepared by subcloning the recombinant mAb-encoding polynucleotides into a suitable expression vector, expressing them in a suitable host (as described below), and isolating the recombinant mAbs.

The present invention provides recombinant expression vectors which include the synthetic, genomic, or cDNA-derived nucleic acid fragments of the invention, i.e. polynucleotides encoding the mAbs of the invention. The nucleotide sequence coding for any of the mAbs of the invention can be inserted into an appropriate expression vector, i.e., a vector that contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translational signals can also be supplied by the native or source gene and/or its flanking regions.

A variety of host vector systems may be utilized to express the recombinant expression vectors of the invention. These include, but are not limited to, mammalian cell systems infected with recombinant virus (e.g., vaccinia virus, adenovirus, retroviruses, etc.); mammalian cell systems transfected with recombinant plasmids; insect cell systems infected with recombinant virus (e.g., baculovirus); microorganisms such as yeast containing yeast expression vectors, or bacteria transformed with recombinant bacteriophage DNA, recombinant plasmid DNA, or cosmid DNA (see, for example, Goeddel, 1990).

Useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well-known cloning vector pBR322 (American Type Culture Collection -"ATCC"-, 12301 Parklawn Drive, Rockville, Maryland 20852, United States of America; ATCC Accession No. 37017). These pBR322 "backbone sections," or functionally equivalent sequences, are combined with an

Samsung Bioepis Exhibit No. 1002
Page 8629

appropriate promoter and the structural gene to be expressed. Promoters commonly used in recombinant microbial expression vectors include, but are not limited to, the lactose promoter system (Chang, et al., Nature 275:615), the tryptophan (trp) promoter (Goeddel, et al., 1980, Gene Expression Technology, Volume 185. Academic Press, Inc., San Diego, CA) and the tac promoter, or a fusion between the tac and trp promoters referred to as the trc promoter (Maniatis, 1982, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Particularly preferred promoters include the T7 promoter, which is used in conjunction with host cell expression of a T7 RNA polymerase (see Studier et al. 1990, Meth. Enzymol. 185:60-89), and the trc promoter, which is found in several commercially available vectors, as described below.

Preferred bacterial expression vectors include, but are not limited to, the pET vectors (see Studier et al. 1990, *supra*) and the Trc vectors. Many of the pET vectors are commercially available from Stratagene Cloning Systems (La Jolla, CA). A particularly preferred vector is the pET Trc SO5/NI vector described below (SEQ ID NO:18). A Trc vector, pTrc 99A, is available from Pharmacia. Other Trc vectors include the pSE vectors (Invitrogen, San Diego, CA).

Preferred bacteria for expression of recombinant mAbs include Bacillus subtilis and, most preferably, Escherichia coli. A particularly preferred strain of E. coli is strain W3110 (ATCC designation 27325). Under certain unusual conditions it may be necessary to use standard bacterial genetics methods to prepare derivative strains of W3110, for example, when a contaminating bacteriophage ("phage") is present in the laboratory where the bacterial manipulations are being carried out. Generally, and particularly for large scale preparation of the recombinant anti-KSSKC mAbs of the invention, it is preferred to use unmodified W3110, or another fully characterized strain.

In cases where phage contamination is a problem and disinfection is not practicable or desirable, it is preferred to identify the phage contaminant and to then use a fully

Samsung Bioepis Exhibit No. 1002
Page 8630

characterized bacterial strain having a known mutation rendering the bacterium resistant to the phage. Preferably the mutation is a null mutant for the receptor for the phage. In some instances, however, the generation use of a relatively uncharacterized phage-resistant derivative strain may be acceptable, particularly in small scale experimental work. When such derivative strains are desired, they may be prepared using the methods described below in Example 11.

For most purposes the use of unmodified W3110 or another fully characterized bacterial strain is generally preferred. This is particularly true for the preparation of pharmaceutical agents comprising the recombinant anti-KSSKC mAbs of the invention. This is because of the problems, well known in the art, of using bacterial strains containing uncharacterized or partially characterized mutations for the production of ingredients of pharmaceutical agents.

The recombinant mAbs of the invention may also be expressed in fungal hosts, preferably yeast of the Saccharomyces genus such as S. cerevisiae. Fungi of other genera such as Aspergillus, Pichia or Kluyveromyces may also be employed. Fungal vectors will generally contain an origin of replication from the 2 μm yeast plasmid or another autonomously replicating sequence (ARS), a promoter, DNA encoding a mAb of the invention, sequences directing polyadenylation and transcription termination, and a selectable marker gene. Preferably, fungal vectors will include an origin of replication and selectable markers permitting transformation of both E. coli and fungi.

Suitable promoter systems in fungi include the promoters for metallothionein, 3-phosphoglycerate kinase, or other glycolytic enzymes such as enolase, hexokinase, pyruvate kinase, glucokinase, the glucose-repressible alcohol dehydrogenase promoter (ADH2), the constitutive promoter from the alcohol dehydrogenase gene, ADH1, and others. See, for example, Schena, et al. 1991 Meth. Enzymol. 194:389-398. Secretion signals, such as those directing the secretion of yeast alpha-factor or yeast invertase, can be incorporated into the fungal vector to promote secretion of a soluble

Samsung Bioepis Exhibit No. 1002
Page 8631

recombinant mAb into the fungal growth medium. See Moir, et al., 1991, Meth. Enzymol. 194:491-507.

Preferred fungal expression vectors can be assembled using DNA sequences from pBR322 for selection and replication in bacteria, and fungal DNA sequences, including the ADH1 promoter and the alcohol dehydrogenase ADH1 termination sequence, as found in vector pAAH5 (Ammerer, 1983, Meth. Enzymol. 101:192). The ADH1 promoter is effective in yeast in that ADH1 mRNA is estimated to be 1 - 2% of total poly(A) RNA.

Various mammalian or insect cell culture systems can be employed to express recombinant mAbs. Suitable baculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow, et al., 1988. Examples of suitable mammalian host cell lines include the COS cell of monkey kidney origin, mouse L cells, murine C127 mammary epithelial cells, mouse Balb/3T3 cells, Chinese hamster ovary cells (CHO), human 293 EBNA and HeLa cells, myeloma, and baby hamster kidney (BHK) cells, with myeloma cells, CHO cells, and human 293 EBNA cells being particularly preferred.

Mammalian expression vectors may comprise non-transcribed elements such as origin of replication, a suitable promoter and enhancer linked to the recombinant mAb gene to be expressed, and other 5' or 3' flanking sequences such as ribosome binding sites, a polyadenylation sequence, splice donor and acceptor sites, and transcriptional termination sequences.

The transcriptional and translational control sequences in mammalian expression vector systems to be used in transforming vertebrate cells may be provided by viral sources. For example, commonly used promoters and enhancers are derived from Polyoma virus, Adenovirus, Simian Virus 40 (SV40), and human cytomegalovirus, including the cytomegalovirus immediate-early gene 1 promoter and enhancer (CMV).

Particularly preferred eukaryotic vectors for the expression of recombinant anti-KSSKC mAbs are pAPEX-1 (SEQ ID NO:3 and, more preferably, pAPEX-3p, SEQ ID NO:4. The vector pAPEX-1 is a derivative of the vector pcDNAI/Amp (Invitrogen) which was modified to increase protein expression levels.

Samsung Bioepis Exhibit No. 1002
Page 8632

First, the 3'-untranslated SV40 small-t antigen intron was removed by deletion of a 601 base pair *Xba*I/*Hpa*I fragment since this intron is susceptible to aberrant splicing into upstream coding regions (Evans and Scarpulla, 1989 Gene 84:135; Huang and Gorman, 1990, Molec. Cell Biol. 10:1805). Second, a chimeric adenovirus-immunoglobulin hybrid intron was introduced into the 5'-untranslated region by replacing a 484 base pair *Nde*I-*Not*I fragment with a corresponding 845 base pair *Nde*I-*Not*I fragment from the vector pRc/CMV7SB (Sato et al., 1994, J. Biol. Chem. 269:17267). Finally, to increase plasmid DNA yields from E. coli, the resulting CMV promoter expression cassette was shuttled into the vector pGEM-4Z (Promega Corp. Madison, WI).

The vector pAPEX-3 is a derivative of the vector pDR2 (Clontech Laboratories, Inc. Palo Alto, CA) in which the EBNA gene was first removed by deletion of a 2.4 kb *Cla*I/*Acc*I fragment. The RSV promoter was then replaced with the CMV promoter and the adenovirus/immunoglobulin chimeric intron by exchanging a 450 bp *Mlu*I/*Bam*HI fragment from pDR2 with a 1.0 kb *Mlu*I/*Bam*HI fragment from the vector pAPEX-1. For construction of pAPEX-3P, a 1.7 kb *Bst*BI/*Swa*I fragment containing the HSV tk promoter and hygromycin phosphotransferase (hyg) gene was removed from pAPEX-3 and replaced with a 1.1 kb *Sna*BI/*Nhe*I fragment containing the SV40 early promoter and puromycin acetyltransferase (pac) gene (Morgenstern and Land, 1990, Nucleic Acids Res. 18:3587-3596) plus a 137 bp *Xba*I/*Cla*I fragment containing an SV40 polyadenylation signal from the vector pAPEX-1.

A particularly preferred host cell for the expression of recombinant mAb-encoding inserts in the pAPEX vectors is the human 293 EBNA cell line (Invitrogen, San Diego, CA).

Another preferred eukaryotic vector for the expression of recombinant mAbs is pcDNAI/Amp (Invitrogen Corporation, San Diego, California). The pcDNAI/Amp expression vector contains the human cytomegalovirus immediate-early gene I promoter and enhancer elements, the Simian Virus 40 (SV40) consensus intron donor and acceptor splice sequences, and the SV40 consensus polyadenylation signal. This vector also contains an SV40

Samsung Bioepis Exhibit No. 1002
Page 8633

origin of replication that allows for episomal amplification in cells (e.g., COS cells, MOP8 cells, etc.) transformed with SV40 large T antigen, and an ampicillin resistance gene for propagation and selection in bacterial hosts.

Purified recombinant mAbs are prepared by culturing suitable host/vector systems to express the recombinant mAb translation products of the nucleic acid molecules of the present invention, which are then purified from the culture media or cell extracts of the host system, e.g., the bacteria, insect cells, fungal, or mammalian cells. Fermentation of fungi or mammalian cells that express recombinant mAb proteins containing a histidine tag sequence (a sequence comprising a stretch of at least 5 histidine residues) as a secreted product greatly simplifies purification. Such a histidine tag sequence enables binding under specific conditions to metals such as nickel, and thereby to nickel (or other metal) columns for purification. Recombinant mAbs may also be purified by protein G affinity chromatography (Proudfoot et al., 1992, Protein Express. Purif. 3:368).

Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The methods and materials which are common to various of the examples are as follows.

Materials and Methods

Induction of GN in Mice

Four month old female B10.D2/nSnJ mice averaging approximately 25 gms each were obtained from the Jackson Laboratory, Bar Harbor, ME. Mice were injected with 0.1 mL daily (six days per week) of a 40 mg/mL solution of horse apoferritin (HAF), which was prepared by dilution of a saline solution of HAF (Sigma Chemical Company Catalog No. A-3641) with PBS.

Anti-C5 Monoclonal Antibodies

Monoclonal antibodies that bind to complement component C5 of the mouse were prepared by standard methods as an IgG fraction from supernatants of cultures of hybridoma BB5.1 (Frei, et al., 1987), which was obtained from Dr. Brigitta Stockinger of the National Institute for Medical Research,

Samsung Bioepis Exhibit No. 1002
Page 8634

Mill Hill, London, England.

Histology

Kidneys were subjected to microscopic analysis using standard histochemical staining and immunofluorescence techniques. Periodic Acid Schiff (PAS) staining of 5μ paraffin sections was by standard methods using a HARLECO PAS histochemical reaction set (EM Diagnostic Systems, Gibbstown, NJ, number 64945/93) according to the manufacturer's directions.

Immunofluorescence staining of 5μ cryostat sections was carried out by standard methods using FITC conjugated sheep anti-mouse C3 (Biodesign International, Kennebunk, ME, Catalog No. W90280F) to detect murine complement component C3, or FITC conjugated goat anti-mouse IgG, IgA, and IgM (Zymed Laboratories, South San Francisco, CA, Catalog No. 65-6411) to detect immune complexes.

Urine Assays

Protein and glucose levels were determined by spotting urine samples on CHEMSTRIP 2GP dipsticks (Boehringer Mannheim Diagnostics, Indianapolis, IN, Catalog No. 200743). The detection areas of these strips change color when exposed to urine containing protein or glucose; a lack of color change indicates no detectable protein or glucose is present. The level of analyte in the urine being tested is read out by matching changed colors with color charts supplied by the manufacturer. The urine protein chart shows colors corresponding to trace, 30, 100, and 500 mg/dL.

Cell Lysis Assays

The cell-lysing ability of complement in blood can be determined using hemolytic assays that are performed as follows: Chicken erythrocytes are washed well in GVBS (Rollins, et al., J Immunol 144:3478-3483, 1990, Sigma Chemical Co. St. Louis, MO, catalog No. G-6514) and resuspended to $2x10^8$/mL in GVBS. Anti-chicken erythrocyte antibody (IgG fraction of anti-chicken-RBC antiserum, Intercell Technologies, Hopewell, NJ) is added to the cells at a final concentration of 25 μg/mL and the cells are incubated for 15 min. at 23°C. The cells are washed 2x with GVBS and

Samsung Bioepis Exhibit No. 1002
Page 8635

$5x10^6$ cells are resuspended to 30 µL in GVBS. A 100 µL volume of serum test solution is then added to yield a final reaction mixture volume of 130 µL. As used herein, reference to the serum percentage and/or serum input in these assays indicates the percent serum in the 100 µL volume of serum test solution.

For assays of mouse serum activity, the 100 µL volume of serum test solution contained 50 µL of diluted (in GVBS) mouse serum and 50 µL of human C5 deficient serum (Quidel Corporation, San Diego, CA). For assays of human serum activity, the serum test solution may contain up to 100% human plasma or serum, with hybridoma supernatants and/or GVBS being added to yield the 100 µL volume. For the assays used to screen hybridoma supernatants discussed below in Example 7, each 100µL volume of serum test solution contained 50µL of hybridoma supernatant and 50µL of a 10% solution of human serum in GVBS, yielding a 5% human serum input.

After incubation for 30 min. at 37°C, percent hemolysis was calculated relative to a fully lysed control sample. Hemolysis was determined by spinning the cells down and measuring released hemoglobin in the supernatant as the optical density at 415nm.

A 50% reduction in hemolysis after treatment with the anti-C5 antibodies used in the practice of the invention means that the percent hemolysis after treatment is one half of the percent hemolysis before treatment.

EXAMPLE 1

Anti-C5 Antibodies Inhibit Glomerular Inflammation and Enlargement

This example illustrates that anti-C5 antibodies will inhibit glomerular inflammation and enlargement.

The protocol for these experiments was as follows. GN-induced mice were treated with anti-C5 antibodies or with PBS as a control after 2 weeks of GN induction. Each mouse received 750 µg of anti-C5 monoclonal antibodies in PBS (30 mg/kg in a 25 gm mouse) or an equal volume of PBS alone. The amount injected was from 0.25 to 0.365 mL (the concentration of antibodies in PBS varied), which was administered by intraperitoneal injection once a day, six days a week.

Samsung Bioepis Exhibit No. 1002
Page 8636

After an additional 2 weeks of induction and treatment, the animals were sacrificed and kidneys were harvested and prepared for histological examination as described above. Kidneys were also obtained from age-matched uninduced and untreated control mice.

Figure 1 shows sections of mouse kidneys with a single glomerulus located centrally amidst surrounding interstitium and cross sections of convoluted tubules in each section. As can be seen therein, the kidneys of the GN-induced, PBS-treated mice (Fig. 1B) developed severe crescentic glomerular pathology, including inflammatory glomerular hypercellularity, apparent basement membrane thickening, and glomerular enlargement, while the glomeruli of the GN-induced, anti-C5-treated animals (Figure 1C) were essentially indistinguishable from the glomeruli of the normal healthy kidneys of the uninduced untreated mice (Figure 1A).

Note that in the glomeruli with severe crescentic pathology, the size of the glomerular capillary network (glomerular tuft) is not enlarged, but shows signs of compression by a crescentic-shaped proliferation of epithelial cells and PAS-positive material, and the Bowman's capsule is dramatically enlarged. Also note that in the section of diseased glomerulus shown in Fig. 1B, the capillary network is split in half by a projection of the hypercellular crescentic mass.

The non-inflamed glomerulus of the uninduced untreated mouse shown in Figure 1A is approximately 100 μ in diameter; the inflamed glomerulus of the GN-induced, PBS treated mouse shown in Fig. 1B is approximately 175 μ in diameter; the non-inflamed glomerulus of the GN-induced, anti-C5-treated mouse shown in Fig. 1C is approximately 90 μ in diameter.

EXAMPLE 2

Anti-C5 Antibodies Prevent/Reduce Proteinuria Associated with GN

This example demonstrates that treatment with anti-C5 antibodies results in the prevention/reduction of kidney damage as evidenced by the lack of significant amounts of protein in the urine (i.e. the presence of less than 100mg/dL

Samsung Bioepis Exhibit No. 1002
Page 8637

of protein in the urine).

The protocol for the experiments of this example was the same as that used in the experiments of Example 1. Five PBS-treated, GN-induced mice, 6 anti-C5-treated, GN-induced mice, and 4 age-matched untreated uninduced mice were used in this study. A first set of urine samples was analyzed prior to treatment after the initial 2 week induction period. A second set of urine samples was analyzed after the 2 week treatment period. None of the untreated uninduced control animals had detectable protein in their urine at either of these timepoints.

The results obtained with the GN-induced mice are set forth in Table 1. As shown therein, at the end of the 2 week PBS treatment period, 4 out of the 5 PBS treated (control) animals developed significant proteinuria, i.e., at least 100mg/dL of protein in the urine. The fifth animal (mouse D in Table 1) did not have detectable protein in the urine at either timepoint but, unlike the other mice in the study, was found to have very high levels of glucose in the urine after the 2 week PBS treatment period, suggesting that this animal was physiologically compromised.

In the anti-C5-treated, GN-induced group, the one mouse that developed significant proteinuria at the end of the initial 2 week induction period (mouse 6 in Table 1) improved by the end of the 2 week antibody treatment period. In addition, in contrast to the development of significant proteinuria in 4 out of 5 PBS-treated, GN-induced mice, none of the anti-C5-treated, GN-induced mice exhibited significant proteinuria at the end of the 2 week antibody treatment period.

EXAMPLE 3

Anti-C5 Antibodies Do Not Inhibit Glomerular Immune Complex Deposition

This example demonstrates that anti-C5 antibodies used in the practice of the invention achieve their therapeutic effects even though immune complexes are deposited in the glomeruli of treated animals at equivalent levels to those seen in the glomeruli of PBS-treated animals. The example

Samsung Bioepis Exhibit No. 1002
Page 8638

further illustrates that the mechanism of operation of the anti-C5 antibodies is not through the inhibition of immune complex deposition in the glomerulus.

The protocol used in the experiments of this example was the same as that used in the experiments of Example 1. Immunofluorescence staining as described above was performed on sections from the same kidneys harvested in Example 1.

The results are shown in Figure 2. As can be seen in this figure, equivalent amounts of immune complexes were deposited in the glomeruli of the kidneys of both the PBS-treated, GN-induced mice (Figure 2B) and the anti-C5-treated, GN-induced mice (Figure 2C), but not in the untreated uninduced controls (Figure 2A). Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed immune complex deposits in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in Fig. 2B and Fig. 2C.

EXAMPLE 4

Anti-C5 Antibodies Inhibit C5b-9 Generation

This example demonstrates that the anti-C5 antibodies used in the practice of the invention inhibit C5b-9 generation. C5b-9 generation was assayed in 2 ways: (1) by testing the cell-lysing (hemolytic) ability of blood samples, and (2) by measuring levels of soluble C5b-9 in blood samples.

Fig. 3 shows the results of cell lysis assays performed as described above, with mouse serum added to the percentage indicated on the X axis ("serum input %"). In these assays, serum from GN-induced animals treated with either anti-C5 antibodies in PBS or PBS alone (see above) was assayed at the end of the two week treatment period. Serum from normal, uninduced, uninjected mice ("normal mouse serum") obtained from Sigma Chemical Company (St. Louis, MO, Catalog No. S-3269) was also assayed as an additional control. These results indicate that the anti-C5 monoclonal antibody administered to mice at a dosage of 30 mg/Kg completely blocked the cell lysing ability of mouse blood at serum input levels 4-fold higher than the levels of normal serum that produce maximum hemolysis in the assay.

Samsung Bioepis Exhibit No. 1002
Page 8639

The effects of an anti-C5 monoclonal antibody raised to human C5 was evaluated in circulating human blood. Hybridoma N19/8 (Wurzner, et al., 1991) was obtained from Dr. Otto Götze, Department of Immunology, University of Göttingen, FRG. The C5 monoclonal antibody was prepared following immunization of mice with purified human C5 protein as described in Wurzner, et al., (1991). The hybridoma was propagated in mice, and the monoclonal antibody recovered and purified as an IgG fraction from mouse ascites fluid (Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Current Protocols In Immunology, John Wiley & Sons, New York, 1992).

To carry out these experiments, as well as others described below in Examples 5 and 6, 300 mL of whole human blood was drawn from a healthy human donor and additionally a 1 mL sample was removed as a control sample for later analysis. The blood was diluted to 600 mL by the addition of Ringer's lactate solution containing 10 U/mL heparin. The anti-C5 mAb (30 mg in sterile PBS) was added to the diluted blood to a final concentration of 50 µg/mL (results using test samples obtained in this way are labeled "+anti-C5 sample" in Fig. 4 and Fig. 6). In a control experiment, an equal volume of sterile PBS was added to diluted blood (results using control samples obtained in this way are labeled "-anti-C5 sample" in Fig. 4 and Fig. 6).

The blood was then used to prime the extracorporeal circuit of a COBE CML EXCEL membrane oxygenator cardiopulmonary bypass (CPB) machine (Cobe BCT, Inc., Lakewood, CO) and circulation through the circuit was started. The circuit was cooled to 28°C and circulated for 60 minutes. The circuit was then warmed to 37°C and circulated for an additional 30 minutes, after which time the experiment was terminated. Mechanical circulation of blood in this fashion activates the complement cascade. Samples were taken at several time points.

At each time point an aliquot of blood was taken, and subaliquots were centrifuged to remove all cells and the remaining plasma diluted 1:1 in Quidel sample preservation

Samsung Bioepis Exhibit No. 1002
Page 8640

solution (Quidel Corporation, San Diego, CA) and stored at -80°C for subsequent evaluation of soluble C5b-9 (sC5b-9) generation. Diluted subaliquots of plasma were also frozen for evaluation of C3a generation (see Example 5, below). Undiluted subaliquots of plasma were frozen at -80°C for analysis in hemolytic assays to evaluate the pharmacokinetics of the effects of the anti-C5 antibodies on the cell lysing ability of complement present in the blood (see Example 6, below). These experiments are also discussed in copending US patent application Serial No. 08/217,391, filed March 23, 1994.

sC5b-9 assays were performed before the addition of the antibody or the commencement of the CPB circuit (labeled "Pre Tx" in Fig. 4 and Fig. 6) using undiluted blood (i.e. blood from the 1mL sample taken before the blood was diluted with Ringer's lactate solution -- labeled "undil" in Fig. 4 and Fig. 6) and Ringer's lactate solution diluted blood (labeled "dil" in Fig. 4 and Fig. 6). Samples of Ringer's lactate solution diluted blood to which the antibody had been added (labeled "Post Tx" in Fig. 4 and Fig. 6) were assayed at the times indicated after starting the CPB circuit.

As can be seen in Figure 4, while sC5b-9 levels were more than 4-fold higher in untreated samples after 90 minutes of circulation than before circulation, the anti-C5 antibody completely inhibited C5b-9 generation throughout the 90 minute time course of circulation so that sC5b-9 levels during circulation were essentially equivalent to control, uncirculated samples, at all timepoints.

EXAMPLE 5

Anti-C5 Antibodies Do Not Inhibit C3 Deposition Or Activation

This example demonstrates that treatment with anti-C5 antibodies does not result in the inhibition of the activation of complement component C3 or in the deposition of C3 or its activated fragments in glomeruli.

The deposition of C3, or the fragments generated by its activation (e.g., C3a and C3b), in the glomeruli of GN-induced and GN-uninduced mice was visualized by immunofluorescence

Samsung Bioepis Exhibit No. 1002
Page 8641

staining with a FITC-conjugated sheep anti-mouse C3 antibody preparation using standard methods, as described above. As can be seen in Fig. 5, kidneys of the PBS-treated (Fig. 5B) and the anti-C5 antibody-treated (Fig. 5C) GN-induced mice had roughly equivalent levels of C3 immunoreactive material in the glomeruli, while the uninduced untreated control mice had only traces of C3 immunoreactive material in their kidneys (Fig. 5A).

Note that the print shown in Fig. 5A was overexposed compared to those of Fig. 5B and Fig. 5C to show the very slight levels of reactivity present in normal uninduced kidneys. Kidneys of GN-induced mice harvested after the 2 week induction period, but before treatment, showed C3 immunoreactive materials in the glomeruli, but at lower levels (as indicated by lower fluorescence intensity) than in the kidney sections shown in Fig. 5B and Fig. 5C.

Anti-human C5 antibodies were also tested for possible inhibition of C3 activation in human blood prepared and circulated as described above in Example 4. Activation of complement component C3 was indicated by the presence in the blood of the C3 activation product C3a. C3a assays were performed as follows.

The plasma samples that had previously been diluted in Quidel sample preservation solution and frozen (see Example 4) were assayed for the presence of C3a by using the Quidel C3a EIA kit (Quidel Corporation, San Diego, CA) according to the manufacturers specifications. Concentrations of C3a in the samples is expressed as ng/well as determined by comparison to a standard curve generated from samples containing known amounts of human C3a.

As seen in Fig. 6, the addition of the anti-C5 mAb had no inhibitory effect on the production of C3a during the circulation of human blood in this experiment.

EXAMPLE 6

Pharmacokinetics of Anti-C5 Antibodies

The *in vivo* duration of action of mAb BB5.1, and a Fab' fragment of mAb BB5.1 (prepared by standard methods) was determined in normal female BALB/cByJ mice (averaging

Samsung Bioepis Exhibit No. 1002
Page 8642

approximately 20 gms each) which were obtained from the Jackson Laboratory, Bar Harbor, ME. The mice were given a single intravenous injection (at 35 mg/kg body weight) of the mAB or the Fab' fragment of the mAb (or an equal volume of PBS as a control). Blood samples were collected from the retroorbital plexus at 1, 4, 24, 96, and 144 hours after administration of PBS; 4, 16, and 24 hours after administration of the Fab' fragment of mAb BB5.1; and 4, 24, 48, 72, 96, and 144 hours after administration of intact mAb BB5.1.

Fig. 7A shows the time course of inhibition of the cell-lysing ability of complement in mouse blood (determined, by testing serum obtained from the blood and diluted to 2.5%, as described above) after the in vivo administration of the mAb, the Fab' fragment, or the PBS. As shown in the figure, the mAb almost completely inhibited the hemolytic activity of the blood throughout the 6 day test period. The Fab', however, had a half-life of approximately 24 hours.

In addition to the above experiments, at the end of the 6 day testing period all of the mice were sacrificed. Kidneys, lungs, and livers were harvested and examined by gross inspection, as well as by microscopic examination of stained sections. All of the organs of the anti-C5 antibody treated animals appeared the same as those taken from PBS control treated animals. The overall appearance of the test and control mice was also indistinguishable prior to necropsy.

Anti-human C5 antibodies were also tested for pharmacokinetic properties in circulating human blood as described above in Example 4. As described therein, the hemolysis inhibiting effects of an anti-human C5 monoclonal antibody were assayed over a 90 minute period of circulation. The results of these assays are charted in Fig. 7B, and show that the N19/8 anti-C5 mAb essentially completely inhibited the cell lysing ability of the human blood during the entire 90 minute period of circulation.

The results of these experiments demonstrate that the anti-C5 antibodies will survive in the bloodstream for a substantial period of time, thus making periodic

Samsung Bioepis Exhibit No. 1002
Page 8643

administration practical.

EXAMPLE 7

Preparation of Anti-C5 Monoclonal Antibodies

A monoclonal antibody suitable for use in the practice of the present invention was prepared in accordance with the teachings of Sims, et al., U.S. Patent No. 5,135,916, as follows.

Balb/c mice were immunized three times by intraperitoneal injection with human C5 protein (Quidel Corporation, San Diego, CA, Cat # A403). The first injection contained 100µg of C5 protein in a complete Freund's adjuvant emulsion, the second immunization contained 100µg of C5 protein in an incomplete Freund's adjuvant emulsion, and the third immunization was 100µg of protein in PBS. The mice were injected at roughly 2 month intervals.

Fusions of splenocytes to myeloma cells to generate hybridomas were performed essentially as described in Current Protocols in Immunology (John Wiley & Sons, New York, 1992, pages 2.5.1 to 2.5.17). One day prior to fusion the mice were boosted IV with 100µg of C5 protein. On the day of fusion, the immunized mice were sacrificed and spleens was harvested. SP2/0-AG14 myeloma cells (ATCC CRL#1581) were used as the fusion partner. SP2/0-AG14 cultures were split on the day before the fusion to induce active cell division. A ratio of 1:10 (myeloma cells:splenocytes) was used in the fusions.

The cells were fused using PEG 1450 in PBS without calcium (Sigma Chemical Company, St. Louis, MO, Catalog No. P-7181) and plated at 1-2.5 x 10^5 cells per well. Selection in EX-CELL 300 medium (JRH Biosciences, Lexena, KS, Catalog No. 14337-78P) supplemented with 10% heat inactivated fetal bovine serum (FBS); glutamine, penicillin and streptomycin (GPS); and HAT (Sigma Chemical Company, St. Louis, MO, Catalog No. H-0262) was started the following day. The fusions were then fed every other day with fresh FBS, GPS, and HAT supplemented medium. Cell death could be seen as early as 2 days and viable cell clusters could be seen as early as 5 days after initiating selection. After two weeks of selection in HAT, surviving hybridomas chosen for further study were transferred

Samsung Bioepis Exhibit No. 1002
Page 8644

to EX-CELL 300 medium supplemented with FBS, GPS, and HT (Sigma Chemical Company, St. Louis, MO, Catalog No. H-0137) for 1 week and then cultured in EX-CELL 300 medium supplemented with FBS and GPS.

Hybridomas were screened for reactivity to C5 and inhibition of complement-mediated hemolysis 10-14 days after fusion, and were carried at least until the screening results were analyzed. The screen for inhibition of hemolysis was the chicken erythrocyte lysis assay described above. The screen for C5 reactivity was an ELISA, which was carried out using the following protocol:

A 50 µL aliquot of a 2 µg/mL solution of C5 (Quidel Corporation, San Diego, CA) in sodium carbonate/bicarbonate buffer, pH 9.5, was incubated overnight at 4°C in each test well of a 96 well plate (Nunc-Immuno F96 Polysorp, A/S Nunc, Roskilde, Denmark). The wells were then subjected to a wash step. (Each wash step consisted of three washes with TBST.) Next, test wells were blocked with 200 µL of blocking solution, 1% BSA in TBS (BSA/TBS), for 1 hour at 37°C. After an additional wash step, a 50 µL aliquot of hybridoma supernatant was incubated in each test well for 1 hour at 37°C with a subsequent wash step. As a secondary (detection) antibody, 50 µL of a 1:2000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG in BSA/TBS, was incubated in each test well for 1 hour at 37°C, followed by a wash step. Following the manufacturer's procedures, 10 mg of O-phenylenediamine (Sigma Chemical Company, St. Louis, MO, Catalog No. P-8287) was dissolved in 25 mLs of phosphate-citrate buffer (Sigma Chemical Company, St. Louis, MO, Catalog No. P-4922), and 50 µL of this substrate solution was added to each well to allow detection of peroxidase activity. Finally, to stop the peroxidase detection reaction, a 50 µL aliquot of 3N hydrochloric acid was added to each well. The presence of antibodies reactive with C5 in the hybridoma supernatants was read out by a spectrophotometric OD determination at 490 nm.

The supernatant from a hybridoma designated as 5G1.1 tested positive by ELISA and substantially reduced the cell-lysing ability of complement present in normal human blood in

Samsung Bioepis Exhibit No. 1002
Page 8645

the chicken erythrocyte hemolysis assay. Further analyses revealed that the 5G1.1 antibody reduces the cell-lysing ability of complement present in normal human blood so efficiently that, even when present at roughly one-half the molar concentration of human C5 in the hemolytic assay, it can almost completely neutralize serum hemolytic activity.

Immunoblot analysis was undertaken to further characterize the 5G1.1 mAb. Human C5 (Quidel Corporation, San Diego, CA, Catalog No. A403) was subjected to polyacrylamide gel electrophoresis under reducing conditions, transferred to a nitrocellulose membrane, and probed with the 5G1.1 mAb as a purified IgG preparation. Two bands were immunoreactive with the 5G1.1 mAb at apparent molecular weights corresponding to those of the alpha and beta chains of the human C5 protein. The two 5G1.1 immunoreactive bands seen on this Western blot were subsequently found to result from the binding of the 5G1.1 antibody to the 115 kDa C5 alpha chain and to a large fragment of the alpha chain that had the same apparent molecular weight (approximately 75 kDa) as the beta chain of C5 and was present in the C5 preparations used for the experiment.

Assays were performed to determine the relative activity of the N19/8 mAb discussed in Examples 4 and 5 with the 5G1.1 mAb in functional hemolytic assays and to assess whether these mAbs blocked the cleavage of C5 to yield C5a. To this end, the N19/8 and 5G1.1 mAbs were directly compared in human complement hemolytic and C5a release assays.

Hemolytic assays performed in the presence of 20% v/v human serum revealed that the 5G1.1 mAb effectively blocked serum hemolytic activity at a final concentration of 6.25µg/ml (0.5 / 1 molar ratio of 5G1.1 / C5) whereas the N19/8 mAb blocked at a higher concentration of 25.0µg/ml (2.0 / 1 molar ratio of N19/8 / C5). When the supernatants from these assays were tested for the presence of C5a, the 5G1.1 mAb was found to have effectively inhibited C5a generation at doses identical to those required for the blockade of C5b-9 mediated hemolytic activity.

In contrast, the N19/8 mAb was 10 fold less effective in

Samsung Bioepis Exhibit No. 1002
Page 8646

blocking the release of C5a in these assays when compared to the 5G1.1 mAb. Furthermore, the ability of the N19/8 mAb to block complement mediated hemolysis was not equivalent to its capacity to block C5a generation in that a dose of 25µg/ml of N19/8 completely blocked hemolysis while only reducing C5a generation by 37%.

Hybridoma 5G1.1 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, United States of America, on April 27, 1994, and has been assigned the designation HB-11625. This deposit were made under the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure (1977).

EXAMPLE 8

Determination of the affinity constants (K_D) for the anti-human C5 monoclonal antibodies 5G1.1 and N19/8

The procedure utilized to determine the dissociation constant (K_D) of antibody-antigen equilibria in solution was that described by Friguet et al., J. Immunol. Meth. 1985, 77:305-319. This method was used to determine the K_D for the anti-human C5 monoclonal antibodies N19/8 and 5G1.1. The monoclonal antibodies were incubated with the antigen (C5) in solution until the equilibrium was reached. The proportion of antibody that remains unbound (free) at equilibrium was measured using a conventional Enzyme Linked Immunosorbant Assay (ELISA). The experimental values of K_D obtained by this method have been shown to be equivalent to those obtained by other methods (immunoprecipitation of the radiolabeled antigen and fluorescence transfer). This method offers the advantage of dealing with unmodified antigen.

Figures 8 and 9 show the Scatchard plots of the binding of the anti-human C5 monoclonal antibodies 5G1.1 and N19/8 to human C5 as measured by ELISA. In each graph (v) represents the fraction of bound antibody and (a) represents the concentration of free antigen at equilibrium. The calculated K_D for the 5G1.1 mAb was 30pM while the calculated K_D for the N19/8 mAb was 43pM. These results indicate that the K_D for

Samsung Bioepis Exhibit No. 1002
Page 8647

the 5G1.1 and N19/8 mAb's are similar, and therefore the functional disparity between the two antibodies cannot be explained simply by the differences in affinity for the C5 antigen.

EXAMPLE 9

Effect of 5G1.1 mAb on Complement Activation During CPB

Experiments involving recirculation of human blood in an CPB circuit, as described above in Examples 4 and 5, were carried out using three doses of the 5G1.1 mAb (15mg, 7.5mg, 3.75mg) as well as controls in the absence of the 5G1.1 mAb. In five such control experiments performed in this series, C3a Fig. 10) and sC5b-9 (Fig. 11) levels increased during the first 30 min and continued to rise throughout the entire experiment. Addition of the 5G1.1 mAb to the CPB circuit had no effect on the generation of C3a in these experiments.

Conversely, addition of the two highest doses (15mg and 7.5mg) of the 5G1.1 mab completely blocked the generation of sC5b-9 in these experiments while the lowest dose (3.75mg) only partially blocked sC5b-9 generation. Hemolytic assays performed on serum samples drawn throughout the time course of these experiments revealed that total serum complement activity was not affected in control experiments (Fig. 12). In contrast, the highest dose of the 5G1.1mAb (15mg) completely blocked complement hemolytic activity, while the two lower doses (7.5mg and 3.75mg), failed to block hemolytic activity.

These results show that the 7.5mg dose effectively blocked C5b-9 generation in the CPB circuit but failed to block C5b-9-mediated hemolytic activity, suggesting that hemolytic assays alone may not accurately reflect the complement activation that occurs during CPB. These results further indicate that the 5G1.1 mAb can completely block complement activation in human blood, as measured by either criterion, at a dosage of 15mg/500ml, a dose that is approximately equivalent to a dose of 150mg for a 70kg patient.

Samsung Bioepis Exhibit No. 1002
Page 8648

EXAMPLE 10

Cloning of Anti-C5 Recombinant Anti-KSSKC Variable Region Genes

Amino Acid Sequencing:

To determine the N-terminal amino acid sequence of the 5G1.1 mAb, a 12% acrylamide gel (37.5:1 acrylamide/N,N'-methylene-bisacrylamide) was prepared and pre-electrophoresed for 45 minutes at 10 mA using 1x pre-electrophoresis buffer (123 mM bis-Tris, pH 6.6, with the cathode buffer reservoir supplemented with 1 mM reduced glutathione). The following day, the pre-electrophoresis buffer in the cathode reservoir was replaced with cathode reservoir buffer (44 mM N-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, 113 mM bis-Tris, 0.1% (w/v) sodium dodecyl sulfate (SDS), 0.067% (w/v) thioglycolic acid) and the pre-electrophoresis buffer in the anode reservoir was replaced with anode reservoir buffer (63 mM bis-Tris, pH 5.9).

75 µg 5G1.1 monoclonal antibody was added to Laemmli sample buffer (30 mM Tris-HCl pH 6.8, 3% (w/v) SDS, 10 mM EDTA, 0.02% (w/v) bromophenol blue, 5% (v/v) glycerol, 2.5% (v/v) beta-mercaptoethanol) and electrophoresed at 10 mA until the bromophenol blue tracking dye reached the bottom of the gel. The protein was transferred to a PROBLOTT membrane (Applied Biosystems, Foster City, CA) using 1X transfer buffer (10 mM cyclohexylaminopropane sulfonic acid, 0.05% (w/v) dithiothreitol, 15% (v/v) methanol) at 50 V for one hour.

Protein bands were localized by staining with 0.2% Ponceau S (in 3% trichloroacetic acid, 3% sulfosalicylic acid) followed by destaining with water. Bands were excised and subjected to amino acid sequence analysis using Edman chemistry performed on a pulsed liquid protein sequencer (ABI model 477A), with the PTH amino acids thereby obtained being analyzed with an on-line microbore HPLC system (ABI model 120A).

To deblock the amino terminus of the 5G1.1 heavy chain, 10 mg 5G1.1 monoclonal antibody was exchanged into reducing buffer (5 M guanidine-HCl, 50 mM Tris-HCl, 10 mM dithiothreitol, pH 8.5) using a PD-10 column (Pharmacia,

Samsung Bioepis Exhibit No. 1002
Page 8649

Piscataway, NJ). After a one hour incubation at room temperature, 50 mM iodoacetamide was added and the incubation allowed to continue for 30 minutes. The carbamidomethylated light and heavy chains thus obtained were separated by size exclusion chromatography on a SUPEROSE 12 (Pharmacia) column equilibrated with 5 M guanidine-HCl, 50 mM Tris-HCl pH 8.5. The carbamidomethylated heavy chain was exchanged into 50 mM sodium phosphate, pH 7.0 using a PD-10 column, subjected to digestion with pyroglutamate aminopeptidase (PanVera, Madison, WI; 0.5 mU per nmol of heavy chain protein), and sequenced as described above.

For determination of internal amino acid sequence, the carbamidomethylated 5G1.1 light chain was exchanged into 2 M urea, 25 mM Tris-HCl, 1 mM EDTA, pH 8.0 and incubated with endoproteinase Lys-C (Promega, Madison, WI; protease:protein ratio of 1:40) at 37°C overnight. The digested material was run on a C18 reversed phase HPLC column (Beckman Instruments, Fullerton, CA) and eluted using a linear 0-50% acetonitrile gradient in 0.1% trifluoroacetic acid. Peaks were subjected to amino acid sequence analysis as described above.

PCR Cloning:

Cloning of the 5G1.1 variable heavy region was performed using a set of commercially available primers (Mouse Ig-PRIMER SET, catalogue number 69831-1, Novagen, Madison, WI). Total RNA was isolated from 5G1.1 hybridoma cells using the acid/guanidinium thiocyanate technique (Chomczynski and Sacchi, Anal. Biochem. 1987, 162:156-159). For first strand cDNA synthesis, ten micrograms total RNA were denatured at 65°C for 5 min., chilled on ice, and added to a 100 µl reaction containing 10 mM Tris pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 10 mM dithiothreitol, 250 µM each dNTP, 20 units AMV reverse transcriptase (Seikagaku America, Rockville, MD), and 10 pmole of the appropriate 3' primer (as described in the Ig-PRIMER SET kit protocol). After incubation at 37°C for one hour, five microliters of the cDNA synthesis reaction were added to a 100 microliter PCR reaction containing: 10 mM Tris-HCl pH 9.0 at 25°C, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% (w/v) gelatin, 1.0% (v/v) Triton® X-100, 200µM each dNTP, 2.5U

Samsung Bioepis Exhibit No. 1002
Page 8650

AMPLITAQ DNA polymerase (Perkin-Elmer-Cetus, Norwalk, CT) and 25 pmoles of the appropriate 5' and 3' primers (as described in the Ig-PRIMER SET kit protocol). The reaction conditions were 1 minute at 95°C, 1 minute at 42°C, and 1 minute at 72°C for 30 cycles, followed by a final extension at 72°C for 10 minutes.

PCR products having the expected size (approximately 450 bp) were cloned into the vector pCRII (Invitrogen, San Diego, CA) using a T/A cloning kit (Invitrogen). DNA sequence analysis of cloned DNA fragments was performed by the dideoxy chain-termination method using double-stranded plasmid DNA as a template. A unique heavy chain variable region was isolated by this procedure, with the resulting plasmid designated p5G1.1 VH 2-1-3. Several clones obtained from independent replicate PCR reactions were sequenced to detect any mutations introduced during the PCR amplification of this variable region.

To clone the 5G1.1 light chain variable region, PCR primers were designed by using the UWGCG program TFASTA (University of Wisconsin, Madison, WI) to search the GenBank rodent subdirectory with the 19mer query amino acid sequence Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr, that was obtained by amino acid sequencing as described above. An exact match to this sequence was located in the murine germline gene encoding the v-kappa k2 variable region (Seidman et al. Proc. Natl. Acad. Sci. USA 1978 75:3881-3885). The DNA sequence of this germline gene was used to design the oligonucleotide UDEC690 (SEQ ID NO:5) for use as a variable region 5'-primer. A murine kappa gene constant region primer, UDEC395 (SEQ ID NO:6) was also synthesized and used in this reaction. Cloning of the 5G1.1 variable light region was performed using the UDEC690 variable region 5'-primer and the UDEC395 murine kappa gene constant region primer.

PolyA mRNA was isolated from hybridoma 5G1.1. The acid/guanidinium thiocyanate procedure (Chomczynski and Sacchi, *supra*) was used to isolate total RNA, and was followed by oligo(dT)-cellulose chromatography of 1 mg of total RNA.

Samsung Bioepis Exhibit No. 1002
Page 8651

For first strand cDNA synthesis, one microliter of the 25 microliters of oligo(dT)-cellulose eluate (containing approximately 2 micrograms of purified 5G1.1 mRNA) was denatured at 65°C for 5 min., chilled on ice, and incubated in extension buffer (10 mM Tris pH 8.3, 50 mM KCl, 1 mM dithithreitol, 240 µM each dNTP) containing 100 nM UDEC395 (SEQ ID NO:6) and 25 units AMV reverse transcriptase (Seikagaku America, Rockville, MD) at 42°C for one hour. Five microliters of the completed first strand reaction was subjected to PCR amplification using amplification buffer supplemented with 2.5 units AMPLITAQ DNA polymerase (Perkin Elmer, Foster City, CA) and 500 nM each of primer UDEC690 (SEQ ID NO:5) and UDEC395 (SEQ ID NO:6). Amplification was performed using 30 cycles each consisting of 1 minute at 95°C, 1 minute at 52°C, and 1 minute at 72°C, followed by a single ten minute incubation at 72°C.

The resulting PCR product was purified using GENECLEAN according to the manufacturer's directions (Bio 101, La Jolla, CA), digested with *Sse*8387 I and *Hind* III, gel purified, and ligated into the vector Bluescript® II SK+ (Stratagene, La Jolla, CA). Ligated plasmids were transformed into the bacterial strain DH10B by electroporation.

Plasmid DNA was purified from cultures of transformed bacteria by conventional methods including column chromatography using a QUIAGEN-TIP-500 column according to the manufacturer's directions (Quiagen, Chatsworth, CA) and sequenced by the Sanger dideoxy chain termination method using SEQUENASE enzyme (U.S. Biochemical, Cleveland, OH). Clones obtained from a second independent PCR reaction verified that no mutations were introduced during the amplification process. The resulting plasmid containing the cloned variable region was designated SK (+) 690/395. This light chain encoding insert in this plasmid coded for both the N-terminal and internal light chain sequences determined by amino acid sequencing of 5G1.1, as described above.

Samsung Bioepis Exhibit No. 1002
Page 8652

EXAMPLE 11

Construction and Expression of Recombinant mAbs

Recombinant DNA constructions encoding the recombinant mAbs comprising the 5G1.1 CDRs are prepared by conventional recombinant DNA methods including restriction fragment subcloning and overlapping PCR procedures. The resulting recombinant mAb-encoding DNAs include:

(1) one encoding a non-humanized (murine) scFv designated 5G1.1M1 scFv (SEQ ID NO:7), wherein CDR L1 is amino acid residues 28-34 of SEQ ID NO:7, CDR L2 is amino acid residues 52-54 of SEQ ID NO:7, CDR L3 is amino acid residues 93-98 of SEQ ID NO:7, CDR H1 is amino acid residues 156-159 of SEQ ID NO:7, CDR H2 is amino acid residues 179-183 of SEQ ID NO:7, and CDR H3 is amino acid residues 226-236 of SEQ ID NO:7;

(2) one encoding a humanized (CDR grafted) scFv designated 5G1.1 scFv CB (SEQ ID NO:8), wherein CDR L1 is amino acid residues 26-36 of SEQ ID NO:8, CDR L2 is amino acid residues 52-58 of SEQ ID NO:8, CDR L3 is amino acid residues 91-99 of SEQ ID NO:8, CDR H1 is amino acid residues 152-161 of SEQ ID NO:8, CDR H2 is amino acid residues 176-192 of SEQ ID NO:8, H3 is amino acid residues 225-237 of SEQ ID NO:8;

(3) one encoding a chimeric light chain (which can form the light chain portion of an Fab) designated 5G1.1M1 VL HuK (SEQ ID NO:9);

(4) one encoding a chimeric Fd (the heavy chain portion of an Fab) designated 5G1.1M1 VH HuG1 (SEQ ID NO:10);

(5) one encoding a humanized (CDR grafted and framework sequence altered) Fd designated 5G1.1 VH + IGHRL (SEQ ID NO:11), wherein CDR H1 is amino acid residues 26-35 of SEQ ID NO:11, CDR H2 is amino acid residues 50-60 of SEQ ID NO:11, and CDR H3 is amino acid residues 99-111 of SEQ ID NO:11;

(6) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH + IGHRLC (SEQ ID NO:12), CDR H1 is amino acid residues 26-35 of SEQ ID NO:12, CDR H2 is amino acid residues 50-66 of SEQ ID NO:12, and CDR H3 is amino acid residues 99-111 of SEQ ID NO:12;

(7) one encoding a humanized (CDR grafted and framework sequence altered) light chain designated 5G1.1 VL + KLV56 (SEQ

Samsung Bioepis Exhibit No. 1002
Page 8653

ID NO:13), wherein CDR L1 is amino acid residues 26-36 of SEQ ID NO:13, CDR L2 is amino acid residues 52-58 of SEQ ID NO:13, and CDR L3 is amino acid residues 91-99 of SEQ ID NO:13;

(8) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL + KLV56B (SEQ ID NO:14), wherein CDR L1 is amino acid residues 26-36 of SEQ ID NO:14, CDR L2 is amino acid residues 52-58 of SEQ ID NO:14, and CDR L3 is amino acid residues 91-99 of SEQ ID NO:14;

(9) one encoding a humanized (CDR grafted, not framework altered) light chain designated 5G1.1 VL + 012 (SEQ ID NO:15), wherein CDR L1 is amino acid residues 24-34 of SEQ ID NO:15, CDR L2 is amino acid residues 50-56 of SEQ ID NO:15, and CDR L3 is amino acid residues 89-97 of SEQ ID NO:15; and

(10) one encoding a humanized (CDR grafted, not framework altered) Fd designated 5G1.1 VH + IGHRLD (SEQ ID NO:16), wherein CDR H1 is amino acid residues 26-35 of SEQ ID NO:16, CDR H2 is amino acid residues 50-60 of SEQ ID NO:16, and CDR H3 is amino acid residues 99-111 of SEQ ID NO:16.

(11) one encoding a humanized (CDR grafted, not framework altered) scFv designated 5G1.1 scFv D012 (SEQ ID NO:17), wherein CDR L1 is amino acid residues 26-36 of SEQ ID NO:17, CDR L2 is amino acid residues 52-58 of SEQ ID NO:17, CDR L3 is amino acid residues 91-99 of SEQ ID NO:17, CDR H1 is amino acid residues 152-161 of SEQ ID NO:17, CDR H2 is amino acid residues 176-186 of SEQ ID NO:17, and CDR H3 is amino acid residues 225-237 of SEQ ID NO:17;

In accordance with the invention, one each of the various L1, L2 and L3 CDRs discussed in (1) to (11) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one L1, one L2, and one L3 CDR, as part of a recombinant antibody or synthetic peptide antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention).

In accordance with the invention, one each of the various H1, H2 and H3 CDRs discussed in (1) to (11) above may be combined with any of the other light chain CDRs so as to make a set of 3 light chain CDRs comprising one H1, one H2, and one H3 CDR, as part of a recombinant antibody or synthetic peptide

Samsung Bioepis Exhibit No. 1002
Page 8654

antibody (i.e., a synthetic peptide with the sequence of a recombinant peptide of the invention).

In accordance with the invention, matched pairs of the variable regions (e.g., a VL and a VH region) of the various antibody molecules, Fds, and light chains described above may be combined with constant region domains by recombinant DNA or other methods known in the art to form full length antibodies of the invention. Particularly preferred constant regions for this purpose are IgG constant regions, which may be unaltered, or constructed of a mixture of constant domains from IgGs of various subtypes, e.g., IgG1 and IgG 4.

Matched pairs of the Fd and light chain encoding DNAs described immediately above -- i.e. (3) and (4), (5) and (7), (6) and (8), and (6) and (9) -- were subcloned together into the APEX-3P vector, essentially as described below in Example 15 for N19/8. The scFv constructs of (1) and (2) were subcloned into pET Trc SO5/NI using conventional techniques.

Plasmids so obtained were introduced by into the bacterial strain ME2 (pET plasmids) by conventional electroporation, or into human 293 EBNA cells (APEX plasmids) by lipofection using 2-3 microliters of TRANSFECTAM reagent (Promega, Madison, WI) per microgram of DNA according to the manufacturer's directions. Bacterial strains ME1 and ME2 are derivatives of *Escherichia coli* strain W3110 (ATCC designation 27325) prepared as follows.

Preparation of W3110 Derivatives ME1 and ME2:

The non-humanized, non-chimeric murine 5G1.1-scFv "m5G1.1-scFv" -- made up of light chain (3) and Fd (4) -- was expressed in a derivative of *E. coli* K12 strain W3110. This derivative was prepared by inactivating an uncharacterized gene to provide protection against infections by a lytic bacteriophage. *E. coli* strain W3110 is a particularly preferred strain because it is fully characterized and is commonly used for recombinant DNA product fermentations.

A single colony of *E. coli* strain W3110 was grown overnight in L medium at 30°C. The cells were collected by centrifugation and resuspended in 10 mM $MgSO_4$. A total of 0.1 ml of the culture was added to 2.5 ml 0.7% L soft agar at 45°C

Samsung Bioepis Exhibit No. 1002
Page 8655

and quickly poured on an L plate. Fifty microliter aliquots of a plaque purified phage lysate, undiluted, diluted 10^{-2} and diluted 10^{-4}, were spotted onto the agar surface. Phage lysates had previously been filtered through 0.45 µm membranes and stored in sterile tubes with a drop of chloroform at 4°C. The spots were allowed to dry on the soft agar surface and incubated overnight at 37°C.

The next day L plates were spread with 10^9 phage PFU and allowed to dry. Using a sterile, flat toothpick, cells from isolated colonies growing in the zones of phage lysis on the spot plates were streaked for single colonies on the plates spread with 10^9 phage PFU and incubated overnight at 37°C. Single colonies were rechecked for phage resistance by cross-streaking after single colony purification. The cross streak test for phage sensitivity was performed as follows. Fifty µl of phage (10^8 pfu/ml) was spread in a vertical line in the left hand portion of the plate using a Pasteur pipette. Additional phage were tested parallel to the first and to the right. The plate was allowed to dry, and strains to be checked for sensitivity or resistance were spread perpendicular to and across the lines of all phages in a single swath from the left to the right. Resistant strains grow in the area of the phage streaks while sensitive strains lyse.

The phage resistant mutant strain ME1 was tested for phage production after overnight growth in L medium and treatment with the DNA damaging agent, mitomycin C. The strain failed to produce viable phage utilizing a standard plaque assay and *E. coli* W3110 as the phage sensitive indicator strain. These results suggest that strain ME1 does not harbor a resident prophage.

Strain ME2 was constructed by site specific integration of the lambdaDE3 prophage (Studier et al. 1990, Meth. Enzymol. 185:60-89) into the ME1 chromosome. Expression of the T7 RNA polymerase, directed by the prophage, allows expression of target genes cloned into pET vectors (Studier et al.,*supra*) under the control of the T7 promoter in the lysogenized host. Lysogenization was accomplished in a three way infection with

Samsung Bioepis Exhibit No. 1002
Page 8656

lambdaDE3, the lambda helper phage, lambdaB10 and the selection phage, lambdaB482 (Studier et al., *supra*).

lambdaDE3 (*imm*21) was constructed by Studier and colleagues (1990, Meth. Enzymol. 185:60-89) by inserting the T7 RNA polymerase gene behind the *E. coli lacUV5* promoter into the *Bam*HI cloning site of lambdaD69(*imm*21). Since cloning into the *Bam*HI site of lambdaD69 interrupts the integrase gene, lambdaDE3 cannot integrate or excise from the chromosome by itself. The helper phage lambdaB10 provides the integrase function that lambdaDE3 lacks but cannot form a lysogen by itself. The selection phage, lambdaB482, lyses any lambdaDE3 host range mutants that otherwise would be among the surviving cells, but it can neither integrate into susceptible cells nor lyse lambdaDE3 lysogens since it has the same immunity region as lambdaDE3 (*imm*21).

Lysogenization protocol:

Strain ME1 was grown in L medium supplemented with 0.2% maltose and 10 mM $MgSO_4$ at 37°C to a density of approximately 10^8 cells/ml. One µl of ME1 cells were incubated with 2 x 10^8 plaque forming units (pfu) of lambdaDE3 and 10^8 pfu of lambdaB10 and lambdaB482. The host/phage mixture was incubated at 37°C for 20 min to allow phage adsorption to ME1 cells. Several dilutions of the cell/phage suspension were spread on L plates to produce plates containing approximately 30-200 candidate lysogens as isolated colonies. The plates were inverted and incubated at 37°C overnight. Several isolated colonies were screened for the acquisition of the lambdaDE3 prophage as described below.

Verification of lambdaDE3 lysogens:

lambdaDE3 lysogen candidates were tested for their ability to support the growth of the T7 phage 4107, a T7 phage deletion mutant that is completely defective unless active T7 RNA polymerase is provided in *trans*. Only lambdaDE3 lysogens will support the normal growth of the phage in the presence of IPTG (isopropyl-beta-thiogalactopyranoside). The T7 phage produces very large plaques on lambdaDE3 lysogens in the presence of IPTG, while very small plaques are observed in the absence of inducer. The size of the plaque in the absence of

Samsung Bioepis Exhibit No. 1002
Page 8657

IPTG is an indication of the basal level of T7 RNA polymerase expression in the lysogen. Putative lambdaDE3 lysogens were grown in L broth supplemented with 0.2 % maltose and 10 mM $MgSO_4$ at 37°C to a cell density of approximately 10^8 cells/ml. A total of 0.5 ml of cells was centrifuged and the pellet was resuspended in 0.2 ml of a T7 phage lysate containing 2 x 10^4 pfu. The phage was allowed to adsorb for 30 min at 37°C. One-half of suspension (0.1 ml) was added to 3.0 ml of molten top agarose at 47°C and poured onto L plates. The remaining aliquot of cell/phage suspension was poured onto an L plate supplemented with 0.4 mM IPTG to check for induction of T7 RNA polymerase. The plates were inverted and incubated at 37°C overnight.

Strains were also tested for the presence of the lambdaDE3 lysogen by demonstrating that each strain was resistant to infection by the phage lambdaB482, which is in the same immunity group (*imm*21), by the cross streak method described above. A lysogen was chosen with a low basal expression level for protein production from pET vectors. The resulting strain, designated ME2, is phage resistant and overexpresses T7 RNA polymerase in the presence of IPTG.

Purification of Humanized 5G1.1-scFv from E. coli:

The humanized 5G1.1-scFv (h5G1.1-scFv) cDNA construct was cloned into the bacterial expression plasmid pET Trc SO5/NI (SEQ ID NO:18) and transformed into E. coli strain ME1. The resulting strain expressing h5G1.1 scFv was grown at 37°C in 2 liter Applikon glass vessel fermentors containing Terrific Broth (1.2 % (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast extract, 0.4% (v/v) glycerol, 90 mM KPO_4, pH 7.0) supplemented with 100 µg/ml ampicillin. The production of recombinant scFv was induced by the addition of 1 mM IPTG when the $O.D._{550}$ of the culture reached 10. After an additional 3 h incubation at 37°C, the cells were harvested by centrifugation and the cell pellets stored at -80°C.

Cells were resuspended in 1 mM EDTA, pH 5.0 at 10 ml per gram weight and lysed by a single pass through a microfluidizer (Model M110T, Microfluidics Corp., Newton, MA). After centrifugation at 17,500 x g for 15 min, the resulting

Samsung Bioepis Exhibit No. 1002
Page 8658

inclusion body pellet was washed by resuspension in 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 mM NaCl, 0.15% (w/v) deoxycholate at 10 ml per gram inclusion body using a Tekmar POLYTRON. The inclusion bodies were again pelleted by centrifugation at 17,500 x g for 15 min and resuspended in 20 mM Tris-HCl pH 9.0, 8 M urea at 10 ml per g. After stirring for 1 h, the sample was centrifuged at 14,000 x g for 30 min to pellet remaining insoluble material.

The extract supernatant was diluted 10-fold with 20 mM Tris-HCl pH 9.0, 7 M urea, 50 µM cupric sulfate and allowed to stir for at least 16 hours at 4°C to refold the scFv. After addition of Biocryl BPA-1000 (TosoHaas, Montgomeryville, PA) as a flocculating agent at 3 µl per ml, the sample was centrifuged at 15,000 x g for 10 minutes to remove insoluble material. The refolding mixture was exchanged into 20 mM Tris, pH 9.0, 1mM EDTA by diafiltration and concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane (Amicon, Beverly, MA).

The properly refolded scFv was then separated from aggregated material and contaminating proteins by anion exchange chromatography using Q Sepharose® Fast Flow (Pharmacia, Piscataway, NJ). Bound scFv was eluted with 20 mM Tris-HCL pH 9.0, 1 mM EDTA containing a linear NaCl gradient (0 to 0.5 M). The fractions containing the scFv were combined, concentrated by ultrafiltration using a stirred cell fitted with a YM10 membrane, and applied to a Sephacryl® S200 HR 26/100 column (Pharmacia) equilibrated in 20 mM Tris-HCl pH 9.0, 1 mM EDTA, 150 mM NaCl. Fractions containing the scFv were combined, exchanged into phosphate-buffered saline by diafiltration, concentrated by ultrafiltration, filtered through a 0.22 µm Millex®-GV filter (Millipore, Bedford, MA), and stored at 4C.

Purification of m5G1.1-scFv from E. coli:

Frozen bacterial cell paste was thawed and resuspended in 2.5 ml of 1 mM EDTA (pH 5) per gram of cell paste. This suspension of cells was lysed by passage through a Microfluidizer (Microfluidics) with the interaction chamber in line and a backpressure of approximately 18000 psi. The cell

Samsung Bioepis Exhibit No. 1002
Page 8659

lysate was then centrifuged at 10,000 rpm in a JA-10 centrifuge rotor at 4°C for 15 min. The supernatant was decanted and discarded.

The pellet was resuspended in 10 ml of 20 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.15% sodium deoxycholate per gram of pellet. This suspension was centrifuged as above for 10 min. Again the supernatant was decanted and discarded. This detergent washed pellet was then resuspended in 10 ml of 8 M urea, 20 mM Tris-HCl, pH 9, 1 mM EDTA. The suspension was stirred at 4°C for 1 hr. and was then diluted 10 fold with 7 M urea, 20 mM Tris-HCl, pH 9 and stirred at 4°C. $CuSO_4$ was then added to a final concentration of 50 µM and stirring was continued overnight at 4°C.

The majority of contaminating proteins (including incorrectly folded versions of m5G1.1 scFv) were then removed by precipitation by diluting (with stirring) the refolded sample five fold with buffer such that the final concentrations after dilution were 1.4 M urea, 25 mM NaCl, 1 mM EDTA, and 20 mM sodium acetate at 4°C. The pH of the dilution buffer when prepared at room temperature was pH 5.0. Prior to dilution the pH of the dilution buffer is determined at 4°C. After the dilution the pH of the sample was greater than pH 5.5. The pH of the sample was then adjusted with 6 N HCl to the initial pH 5.0 of the buffer. The solution immediately became cloudy and it was left stirring at 4-8°C for 0.5 to 24 hours.

The precipitate was removed by filtering the sample through a 300 kDa cut-off ultrafiltration membrane (Millipore Corporation, Bedford, MA). The permeate was collected and concentrated 5 fold using a 10 kDa cutoff ultrafiltration membrane (Millipore). This concentrated retentate was then diluted 2 fold with 20 mM sodium acetate, 1 mM EDTA, pH 5.0 in order to lower the NaCl concentration to 12.5 mM.

The diluted retentate was then loaded at 4°C onto a SP Sepharose FF column (Pharmacia) equilibrated in 0.7 M urea, 1 mM EDTA, 10 mM NaCl, 20 mM sodium acetate, pH 5.0, at a linear flowrate of 5 cm/min. Bed height was equal to or greater than 3.5 cm. Following loading the column was washed with 40

Samsung Bioepis Exhibit No. 1002
Page 8660

column volumes (CV) of equilibration buffer. The column was then washed with 20 CV of 20 mM sodium acetate, pH 5.0, 1 mM EDTA. The bound scFv was then eluted using 20 mM sodium citrate, pH 5.8, 1 mM EDTA. A single peak was collected in approximately 4 column volumes.

The SP Sepharose eluate was then adjusted to 20 mM Tris-HCl by addition of 1 M Tris-HCl, pH 8. The pH of the sample was adjusted to 8.0 by addition of 1 N NaOH. This sample was loaded onto a Q Sepharose FF column (Pharmacia) equilibrated in 20 mM Tris-HCl, pH 8.0, 1 mM EDTA at room temperature at a flowrate of 5 cm/min. The flow through fraction containing the scFv was collected.

The Q Sepharose flow through fraction was then adjusted to 150 mM NaCl and concentrated to 10 mg of scFv per ml using a 10 kDa cutoff ultrafiltration membrane. This concentrated sample was then loaded onto a Sephacryl S200 column equilibrated in phosphate buffered saline, pH 7.4 and eluted at 0.4 cm/min. The fractions were analyzed by SDS-PAGE and silver staining. Peak fractions were combined after discarding the front and back shoulder fractions that contained the majority of contaminants.

EXAMPLE 12

Functional Analysis of the m5G1.1 scFv

Titration of the m5G1.1 scFv in hemolytic assays revealed that the m5G1.1 scFv inhibited human complement-mediated lysis in a dose dependent fashion (Fig. 13). Direct comparison of the efficacy of the m5G1.1 scFv to the 5G1.1mAb and Fab demonstrated that the m5G1.1 scFv completely blocked C5b-9-mediated hemolysis in 20% human serum at 0.15μM while the 5G1.1 mAb and Fab blocked at 0.06-0.08μM. Analysis of C5a generation in these assays revealed similar results in that the 5G1.1 scFv completely blocked C5a generation at 0.15μM while the 5G1.1 mAb and Fab blocked at 0.06-0.08μM (Fig. 14). Taken together these experiments indicated that unlike N19/8, which lost half of its effectiveness at blocking C5a generation upon being engineered as an scFv (SEQ ID NO:19), the 5G1.1 murine scFv retained the capacity to block the generation of both C5a and C5b-9.

Samsung Bioepis Exhibit No. 1002
Page 8661

Additionally, these data demonstrate that the m5G1.1 scFv retained similar activity to that of the parent molecule (the native murine 5G1.1 mAb) in that the molar concentration of 5G1.1 murine scFv required to completely block C5a and C5b-9 (0.15μM) was within two-fold of that required for the 5G1.1 mAb and Fab (0.06-0.08μM).

In order to determine whether the m5G1.1 scFv retained the capacity to block the activation of complement in the *ex vivo* model of cardiopulmonary bypass, 4.5mg of the purified bacterially produced 5G1.1 murine scFv was added to the CPB circuit and complement activation was monitored. In control experiments, both C3a and C5b-9 levels increased throughout the time course of the experiment. In a single experiment, addition of 4.5mg of the m5G1.1 scFv to the CPB circuit had no effect on the generation of C3a (Fig. 15). Conversely, complement hemolytic activity as well as the generation of sC5b-9 was completely blocked in this experiment (Fig. 16 and Fig. 17).

Example 13

Characterization of the Epitope Recognized by 5G1.1

Tryptic digestion: Twenty micrograms of purified human C5 (Advanced Technologies, San Diego, CA) was subjected to enzymatic digestion with 1 μg of TPCK-treated trypsin (Worthington Biochemical Corp., Freehold, NJ). The digestion was allowed to continue for 3 minutes, after which time it was stopped by the addition of 20 μg soy bean trypsin inhibitor (Worthington). The reaction was then denatured and reduced by the addition of protein sample buffer and immediately boiled for 5 min. The digested fragments were size fractionated through a SDS-PAGE on a 12 % gel. The gel was then electroblotted in transfer buffer (20% (v/v) methanol, 25 mM Tris-base pH 8.0, and 192 mM glycine) to nitrocellulose (Bio-Rad Laboratories, Hercules, CA) and subjected to ECL western blot analysis using either 5G1.1 or a C5a specific monoclonal antibody (G25/2, obtained from Dr. Otto Götze, Department of Immunology, University of Göttingen, Germany).

The filters were incubated twice for 30 minutes each in blocking solution (500 mM NaCl, 5 mM Tris pH 7.4, 10% (v/v)

Samsung Bioepis Exhibit No. 1002
Page 8662

nonfat dry milk, and 0.2% (v/v) Tween-20®). The filters were then changed to fresh blocking solution (20 ml) containing the primary antibody and incubated for 40 minutes on a rocking platform. The filters were rinsed briefly with washing solution (500 mM NaCl, 35 mM Tris pH7.4, 0.1% SDS, 1% NP40, and 0.5% deoxycholic acid) to remove any milk, and then fresh wash solution was added and incubated for two 20 minute intervals on an orbiting shaker. The filters were rinsed briefly with 10 to 20 mls of secondary antibody solution (500 mM NaCl, 5 mM Tris pH 7.4, 10% (v/v) Nonfat dry milk, 0.2% (v/v) Tween-20, and 1% NP-40) and then incubated with fresh secondary antibody solution containing a 1:2000 dilution of HRP conjugated goat anti-mouse for 20 minutes on a rocking platform. The filters were then washed as described above, incubated in ECL® reagent (Amersham Corp., Arlington Heights, IL) for 1 minute and then exposed to ECL® Hyperfilm® (Amersham).

Acid Hydrolysis: Twenty micrograms of purified human C5 (Advanced Technologies) was subjected to hydrolysis in 1N acetic acid. The 20 µg of human C5 (1µg/µl) was added to 20 µl of 2N acetic acid and incubated for 10 min at 100°C. The sample was denatured and reduced with protein sample buffer, also at 100°C, for 5 minutes. The acid was neutralized by dropwise addition of a saturated tris base solution until the sample turned blue. The cleavage products were then size fractionated by SDS-PAGE and western blotted as described above. For N-terminal sequencing, the gel fractionated acid hydrolysate was transferred to PVDF membrane. N-terminal sequence was obtained by excising the 46 kDa acid hydrolysis fragment band from a PVDF membrane and subjecting it to amino acid sequence analysis as discussed above in Example 10.

Deglycosylation: Reduced and denatured acid hydrolyzed or tryptic fragments of human C5 were subjected to deglycosylation with N-Glycosidase F (Peptide-N-Glycosidase F, Boehringer Mannheim Corp., Indianapolis, IN) according to the manufacture's directions.

Results: Acid hydrolysis of human C5 yielded a fragment with an apparent molecular weight by SDS-PAGE of 46 kDa that was immunoreactive for both the anti-C5a mAb G25/2 and the

Samsung Bioepis Exhibit No. 1002
Page 8663

anti-C5 alpha chain mAb 5G1.1. Western blots probed with both antibodies simultaneously, as well as silver stain SDS-PAGE analysis, confirmed the presence of a single 46 kDa fragment that was immunoreactive with both antibodies. The presence of a single immunoreactive fragment containing binding sites for both 5G1.1 and G25/2 strongly suggested that the 5G1.1 epitope was contained within approximately the first 46 kDa of the N-terminus of the alpha chain of C5.

As discussed above in the description of the complement system under the heading "Background Physiology & Pathology," a compound (e.g., an antibody) that binds to a site at or immediately adjacent to the C5a cleavage site would have the potential to act as a terminal complement inhibitor. The potential inhibitory activity of antibodies binding to this site led to the expectation that the C5 alpha chain-binding 5G1.1 antibody would bind to an epitope at or near the C5a cleavage site. The finding that 5G1.1 bound to the 46 kDa acid hydrolysis fragment of C5 lent support to this expectation.

Western blot analysis of the tryptic digestion products identified one proteolytic fragment migrating at approximately 27 kDa that was immunoreactive with 5G1.1. Likewise, one immunoreactive proteolytic fragment migrating at approximately 29 kDa was observed following western blot analysis with the anti-C5a mAb G25/2. Experiments in which a blot was simultaneously probed with both 5G1.1 and G25/2 demonstrated that each band was distinct and that their apparent differential mobility was not a gel anomaly. This was surprising, because the 5G1.1 mAb was thought likely to bind to the C5 convertase cleavage site. 5G1.1 was thus expected to be immunoreactive with any fragment of C5 of over 12 kDa that exhibited immunoreactivity with G25/2. Such a fragment would contain enough of the extreme amino terminus of the C5 alpha chain to bind specifically to the anti-C5a mAb, and enough beyond that to encompass a region including and extending beyond the C5 convertase cleavage site.

The immunoreactivity of G25/2 with the 29 kDa fragment indicated that that fragment contains the N-terminal region of

Samsung Bioepis Exhibit No. 1002
Page 8664

the alpha chain of C5 that is cleaved off to yield C5a. Furthermore, because 5G1.1 was not immunoreactive with this band, the 5G1.1 epitope was not likely to be contained within approximately the first 29 kDa of the N-terminus of the alpha chain of C5, and therefore could not be located near the C5 convertase cleavage site.

These tryptic digestion and acid hydrolysis mapping data suggested that the 5G1.1 epitope was contained within a region starting about 29 kDa (including post-translational modifications) from the N-terminus of the alpha chain of C5 and continuing 17 kDa in a C-terminal direction, i.e., ending 46 kDa from the N-terminus, a surprising finding in view of the expectation, discussed above, that the antibody would bind at or immediately adjacent to the point at which C5a is cleaved off of the C5 alpha chain, i.e., at or immediately adjacent to amino acid residue 733 of SEQ ID NO:2.

Post-translational modifications can alter the mobility of proteins during SDS-PAGE electrophoresis. One such modification is the addition of carbohydrate via N-linked glycosylation. As discussed above under the heading "Background Physiology & Pathology", C5 is glycosylated, as is C5a. C5a is glycosylated at an asparagine residue corresponding to amino acid number 723 of the full length pro-C5 precursor of human C5 (SEQ ID NO:2).

Computer analysis of the human C5 alpha chain suggests potential N-linked glycosylation sites at positions corresponding to amino acid numbers 893, 1097, and 1612 of SEQ ID NO:2. In order to determine the contribution of carbohydrate to the electrophoretic mobility of both the tryptic and acid fragments, enzymatic deglycosylation of the fragments was performed and followed by western blot analysis. It was determined that each tryptic fragment lost approximately 3 kDa in apparent molecular weight while the acid fragment lost approximately 6 kDa.

This result was interpreted as indicating that the tryptic fragments were each glycosylated at a single site and that the 46 kDa acid fragment was glycosylated at two sites (one of which was the known glycosylation site in C5a referred to

Samsung Bioepis Exhibit No. 1002
Page 8665

above). The diminished mobility observed following deglycosylation agrees with the computed prediction of a second N-linked glycosylation site within the first 233 amino acids of the C5 alpha chain.

N-terminal sequence analysis determined that the first four amino acids of the 46 kDa fragment generated by 1N acetic acid treatment was Thr Leu Gln Lys. This sequence is found only once in the full length human pro-C5 precursor molecule - at a position corresponding to amino acids 660 through 663 of SEQ ID NO:2. This four amino acid sequence also corresponds to the sequence of the amino-terminus of the alpha chain of human C5 and, thus to the amino-terminus of human C5a.

In order to more precisely map the binding site of 5G1.1, overlapping peptide analysis was performed. The sequence predicted to be contained within the 17 kDa section of human C5 described above (SEQ ID NO:2; amino acids 893 through 1019) together with an extension of 43 amino acids towards the N-terminus and 30 amino acids towards the C-terminus (a total of 200 amino acids) was synthesized as a series of 88 overlapping peptides by solid phase synthesis on polypropylene filters (Research Genetics Inc., Huntsville, AL).

The 43 and 30 amino acid extensions were added to allow for possible inaccuracies in the prediction of the span of this 17 kDa region. Such inaccuracies are likely due to the uncertainty of the specific extent of glycosylation of each of the various regions of C5a, as well as to the aberrant gel mobility that is commonly seen when highly charged polypeptides (such as the 5G46k fragment and the 5G27k fragment) are analyzed by SDS-PAGE. As discussed above in the Summary of the Invention, a 200 amino acid peptide corresponding to the region covered by these overlapping peptides is referred to herein as the "5G200aa" peptide.

Because of the expectation that the 5G1.1 antibody would bind at the C5a cleavage site, an additional set of 8 overlapping peptides was synthesized that spanned a 30 amino acid section spanning the C5a cleavage site (amino acids 725 through 754 of SEQ ID NO:2). A peptide having the sequence of

Samsung Bioepis Exhibit No. 1002
Page 8666

this 30 amino acid section is referred to herein as the "cleavage site peptide". A 325aa peptide spanning amino acid residues 725-1049 of SEQ ID NO:2 (this peptide spans the region covered by the cleavage site peptide and the 5G200aa peptide) is referred to herein as the "5G325aa" peptide.

These filters were probed with 5G1.1 as described above for ECL western blot analysis, and a set of 4 overlapping peptides spanning the region corresponding to amino acid residues 3-19 of the KSSKC peptide (SEQ ID NO:1) each gave a positive signal indicative of monoclonal antibody binding, while peptides corresponding to the C5a cleavage site did not bind to the 5G1.1 antibody.

EXAMPLE 14

C3/C4 Binding Assay

C3 and C4 are both key components of classical C5 convertase, and C3 is also a key component of alternative C5 convertase. These C5 convertases are required for the conversion of C5 to active C5a and C5b. The ability to block C5 binding to C3 and C4 is thus a desirable property for an antibody to be used in treatment of complement mediated diseases in accordance with the present invention.

96 well microtiter plates were coated with 50µl/well, 10µg/ml of either purified human C3 or C4 (Quidel) for 1 hour at 37°C. The plates were then blocked with 200µl/well of TBS containing 1% BSA for 1 hour at room temperature. After three washes in TBS .1% BSA, purified human C5 (Quidel, 20µg/ml in TBS 1% BSA) was added to the plates in the presence (20µg/ml) or absence of a 5G1.1 Fab (derived from 5G1.1 by conventional papain digestion) and allowed to incubate for 1 hour at 37°C. After three washes in TBS/.1% BSA, a monoclonal antibody directed against the C5 beta chain (N19/8, 5µg/ml) was added to the wells to detect C5 bound to either C3 or C4. After three final washes in TBS/.1% BSA, the plate was developed using a horseradish peroxidase-conjugated secondary antibody and the appropriate substrate.

The results of these assays showed that the 5G1.1 mAb inhibited the binding of purified human C5 to either C3 or C4 by at least 60% to 90%. As used herein and in the claims,

Samsung Bioepis Exhibit No. 1002
Page 8667

such a 60% to 90% reduction in C3 or C4 binding is a "substantial reduction" in C3 or C4 binding.

EXAMPLE 15

Construction and Functional Analysis of N19/8 Chimeric Fab

The heavy chain and light chain variable regions from the hybridoma N19-8 were cloned by PCR using the Ig-Prime System (Novagen) as described by the manufacturer. Clones from multiple independent PCR reactions were sequenced to detect mutations introduced during the PCR amplification. An N19-8 VL/human kappa constant region chimeric cDNA was created by using a plasmid containing the N19-8 light chain variable region and the plasmid pHuCK (Hieter et al., 1980 Cell, 22:197-207) as templates in an overlapping PCR reaction.

Similarly, an N19-8 VH/human IgG1 Fd chimeric cDNA was created using a plasmid containing the N19-8 heavy chain variable region and a plasmid containing the human IgG1 gene (obtained from Ilan R. Kirsch, National Cancer Institute, Bethesda, MD) as templates. This Fd construct contained the first nine amino acids of the IgG1 hinge region, including the cysteine residue which normally forms a disulfide bond with the terminal cysteine residue of the kappa light chain.

The resulting chimeric cDNAs were separately cloned into the APEX-1 vector using appropriate flanking restriction enzyme sites introduced during the PCR amplification procedure and sequenced. A fragment containing the promoter, intron, and cDNA insert from one of these APEX vectors was subsequently subcloned into the polylinker of the other to produce a single vector directing the expression of both the light chain and Fd. The tandem expression cassette from this APEX-1 vector was subsequently subcloned into APEX-3P, which was transfected into 293 EBNA cells for expression of the chimeric Fab.

When tested for the ability to block complement hemolytic activity and C5a generation, the chimeric N19/8 Fab retained the ability to block hemolytic activity, but lost 50% of its C5a generation blocking capacity.

Samsung Bioepis Exhibit No. 1002
Page 8668

Throughout this application various publications and patent disclosures are referred to to more fully describe the state of the art to which the present invention pertains.

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention as defined by the following claims.

Samsung Bioepis Exhibit No. 1002
Page 8669

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Samsung Bioepis Exhibit No. 1002
Page 8670

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Samsung Bioepis Exhibit No. 1002
Page 8671

TABLE 1

Prevention/Reduction of Proteinuria by Treatment With Anti-C5 Antibodies

	Before Treatment	After Treatment
	Urine Protein (mg/dL)	Urine Protein (mg/dL)
PBS Control		
mouse A	none	100
mouse B	none	500
mouse C	none	500
mouse D*	trace	trace
mouse E	100	100
Anti-C5 Treated		
mouse 1	none	none
mouse 2	none	30
mouse 3	30	trace
mouse 4	30	30
mouse 5	30	30
mouse 6	100	30

* Mouse D had more than 500mg/dL urine glucose after treatment

Samsung Bioepis Exhibit No. 1002
Page 8672

SEQUENCE LISTING

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(A) MEDIUM TYPE: 3.5 inch, 1.4Mb storage
(B) COMPUTER: Macintosh Cetris 610
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(D) SOFTWARE: WordPerfect 3.0
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(A) APPLICATION NUMBER:
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Samsung Bioepis Exhibit No. 1002
Page 8673

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(2) INFORMATION FOR SEQ ID NO:1:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: Amino Acid
(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(A) DESCRIPTION: KSSKC peptide
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Val Ile Asp His Gln Gly Thr Lys Ser Ser
5 10

Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
15 20

(2) INFORMATION FOR SEQ ID NO:2:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1658 Amino Acids
(B) TYPE: Amino Acid

Samsung Bioepis Exhibit No. 1002
Page 8674

(C) STRANDEDNESS: Single
(D) TOPOLOGY: Linear
(A) DESCRIPTION: Pro-C5 Polytpeptide
(iii) HYPOTHETICAL: No
(iv) ANTIISENSE: No
(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(x) PUBLICATION INFORMATION:
(A) AUTHORS: Haviland, D.L.
Haviland, J.C.
Fleischer, D.T.
Hunt, A.
Wetsel, R.A.

(B) TITLE: Complete cDNA Sequence of Human Complement Pro-C5
(C) JOURNAL: Journal of Immunology
(D) VOLUME: 146
(F) PAGES: 362-368
(G) DATE: 1991

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Leu Leu Gly Ile Leu Cys Phe Leu
-15 -10

Ile Phe Leu Gly Lys Thr Trp Gly Gln Glu Gln Thr Tyr Val
-5 -1 5

Ile Ser Ala Pro Lys Ile Phe Arg Val Gly Ala Ser Glu Asn
10 15 20

Ile Val Ile Gln Val Tyr Gly Tyr Thr Glu Ala Phe Asp Ala
25 30

Thr Ile Ser Ile Lys Ser Tyr Pro Asp Lys Lys Phe Ser Tyr

Samsung Bioepis Exhibit No. 1002
Page 8675

35 40 45

Ser Ser Gly His Val His Leu Ser Ser Glu Asn Lys Phe Gln
50 55 60

Asn Ser Ala Ile Leu Thr Ile Gln Pro Lys Gln Leu Pro Gly
65 70 75

Gly Gln Asn Pro Val Ser Tyr Val Tyr Leu Glu Val Val Ser
80 85 90

Lys His Phe Ser Lys Ser Lys Arg Met Pro Ile Thr Tyr Asp
95 100

Asn Gly Phe Leu Phe Ile His Thr Asp Lys Pro Val Tyr Thr
105 110 115

Pro Asp Gln Ser Val Lys Val Arg Val Tyr Ser Leu Asn Asp
120 125 130

Asp Leu Lys Pro Ala Lys Arg Glu Thr Val Leu Thr Phe Ile
135 140 145

Asp Pro Glu Gly Ser Glu Val Asp Met Val Glu Glu Ile Asp
150 155 160

His Ile Gly Ile Ile Ser Phe Pro Asp Phe Lys Ile Pro Ser
165 170

Asn Pro Arg Tyr Gly Met Trp Thr Ile Lys Ala Lys Tyr Lys
175 180 185

Glu Asp Phe Ser Thr Thr Gly Thr Ala Tyr Phe Glu Val Lys
190 195 200

Glu Tyr Val Leu Pro His Phe Ser Val Ser Ile Glu Pro Glu
205 210 215

Samsung Bioepis Exhibit No. 1002
Page 8676

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Tyr Asn Phe Ile Gly Tyr Lys Asn Phe Lys Asn Phe Glu Ile
            220                 225                 230

Thr Ile Lys Ala Arg Tyr Phe Tyr Asn Lys Val Val Thr Glu
                235                 240

Ala Asp Val Tyr Ile Thr Phe Gly Ile Arg Glu Asp Leu Lys
245                 250                 255

Asp Asp Gln Lys Glu Met Met Gln Thr Ala Met Gln Asn Thr
    260                 265                 270

Met Leu Ile Asn Gly Ile Ala Gln Val Thr Phe Asp Ser Glu
        275                 280                 285

Thr Ala Val Lys Glu Leu Ser Tyr Tyr Ser Leu Glu Asp Leu
            290                 295                 300

Asn Asn Lys Tyr Leu Tyr Ile Ala Val Thr Val Ile Glu Ser
                305                 310

Thr Gly Gly Phe Ser Glu Glu Ala Glu Ile Pro Gly Ile Lys
315                 320                 325

Tyr Val Leu Ser Pro Tyr Lys Leu Asn Leu Val Ala Thr Pro
    330                 335                 340

Leu Phe Leu Lys Pro Gly Ile Pro Tyr Pro Ile Lys Val Gln
        345                 350                 355

Val Lys Asp Ser Leu Asp Gln Leu Val Gly Gly Val Pro Val
            360                 365                 370

Ile Leu Asn Ala Gln Thr Ile Asp Val Asn Gln Glu Thr Ser
                375                 380

Asp Leu Asp Pro Ser Lys Ser Val Thr Arg Val Asp Asp Gly
385                 390                 395
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Samsung Bioepis Exhibit No. 1002
Page 8677

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Val Ala Ser Phe Val Leu Asn Leu Pro Ser Gly Val Thr Val
    400                 405                 410

Leu Glu Phe Asn Val Lys Thr Asp Ala Pro Asp Leu Pro Glu
        415                 420                 425

Glu Asn Gln Ala Arg Glu Gly Tyr Arg Ala Ile Ala Tyr Ser
            430                 435                 440

Ser Leu Ser Gln Ser Tyr Leu Tyr Ile Asp Trp Thr Asp Asn
                445                 450

His Lys Ala Leu Leu Val Gly Glu His Leu Asn Ile Ile Val
455                 460                 465

Thr Pro Lys Ser Pro Tyr Ile Asp Lys Ile Thr His Tyr Asn
    470                 475                 480

Tyr Leu Ile Leu Ser Lys Gly Lys Ile Ile His Phe Gly Thr
        485                 490                 495

Arg Glu Lys Phe Ser Asp Ala Ser Tyr Gln Ser Ile Asn Ile
            500                 505                 510

Pro Val Thr Gln Asn Met Val Pro Ser Ser Arg Leu Leu Val
                515                 520

Tyr Tyr Ile Val Thr Gly Glu Gln Thr Ala Glu Leu Val Ser
525                 530                 535

Asp Ser Val Trp Leu Asn Ile Glu Glu Lys Cys Gly Asn Gln
    540                 545                 550

Leu Gln Val His Leu Ser Pro Asp Ala Asp Ala Tyr Ser Pro
        555                 560                 565
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Samsung Bioepis Exhibit No. 1002
Page 8678

```
Gly Gln Thr Val Ser Leu Asn Met Ala Thr Gly Met Asp Ser
            570                 575                 580

Trp Val Ala Leu Ala Ala Val Asp Ser Ala Val Tyr Gly Val
                585                 590

Gln Arg Gly Ala Lys Lys Pro Leu Glu Arg Val Phe Gln Phe
595                 600                 605

Leu Glu Lys Ser Asp Leu Gly Cys Gly Ala Gly Gly Gly Leu
    610                 615                 620

Asn Asn Ala Asn Val Phe His Leu Ala Gly Leu Thr Phe Leu
        625                 630                 635

Thr Asn Ala Asn Ala Asp Asp Ser Gln Glu Asn Asp Glu Pro
            640                 645                 650

Cys Lys Glu Ile Leu Arg Pro Arg Arg Thr Leu Gln Lys Lys
                655                 660

Ile Glu Glu Ile Ala Ala Lys Tyr Lys His Ser Val Val Lys
665                 670                 675

Lys Cys Cys Tyr Asp Gly Ala Cys Val Asn Asn Asp Glu Thr
    680                 685                 690

Cys Glu Gln Arg Ala Ala Arg Ile Ser Leu Gly Pro Arg Cys
        695                 700                 705

Ile Lys Ala Phe Thr Glu Cys Cys Val Val Ala Ser Gln Leu
            710                 715                 720

Arg Ala Asn Ile Ser His Lys Asp Met Gln Leu Gly Arg Leu
                725                 730

His Met Lys Thr Leu Leu Pro Val Ser Lys Pro Glu Ile Arg
735                 740                 745
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Samsung Bioepis Exhibit No. 1002
Page 8679

```
Ser Tyr Phe Pro Glu Ser Trp Leu Trp Glu Val His Leu Val
    750                 755                 760

Pro Arg Arg Lys Gln Leu Gln Phe Ala Leu Pro Asp Ser Leu
        765                 770                 775

Thr Thr Trp Glu Ile Gln Gly Ile Gly Ile Ser Asn Thr Gly
            780                 785                 790

Ile Cys Val Ala Asp Thr Val Lys Ala Lys Val Phe Lys Asp
                795                 800

Val Phe Leu Glu Met Asn Ile Pro Tyr Ser Val Val Arg Gly
805                 810                 815

Glu Gln Ile Gln Leu Lys Gly Thr Val Tyr Asn Tyr Arg Thr
    820                 825                 830

Ser Gly Met Gln Phe Cys Val Lys Met Ser Ala Val Glu Gly
        835                 840                 845

Ile Cys Thr Ser Glu Ser Pro Val Ile Asp His Gln Gly Thr
            850                 855                 860

Lys Ser Ser Lys Cys Val Arg Gln Lys Val Glu Gly Ser Ser
                865                 870

Ser His Leu Val Thr Phe Thr Val Leu Pro Leu Glu Ile Gly
875                 880                 885

Leu His Asn Ile Asn Phe Ser Leu Glu Thr Trp Phe Gly Lys
    890                 895                 900

Glu Ile Leu Val Lys Thr Leu Arg Val Val Pro Glu Gly Val
        905                 910                 915
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Samsung Bioepis Exhibit No. 1002
Page 8680

Lys Arg Glu Ser Tyr Ser Gly Val Thr Leu Asp Pro Arg Gly
920 925 930

Ile Tyr Gly Thr Ile Ser Arg Arg Lys Glu Phe Pro Tyr Arg
935 940

Ile Pro Leu Asp Leu Val Pro Lys Thr Glu Ile Lys Arg Ile
945 950 955

Leu Ser Val Lys Gly Leu Leu Val Gly Glu Ile Leu Ser Ala
960 965 970

Val Leu Ser Gln Glu Gly Ile Asn Ile Leu Thr His Leu Pro
975 980 985

Lys Gly Ser Ala Glu Ala Glu Leu Met Ser Val Val Pro Val
990 995 1000

Phe Tyr Val Phe His Tyr Leu Glu Thr Gly Asn His Trp Asn
1005 1010

Ile Phe His Ser Asp Pro Leu Ile Glu Lys Gln Lys Leu Lys
1015 1020 1025

Lys Lys Leu Lys Glu Gly Met Leu Ser Ile Met Ser Tyr Arg
1030 1035 1040

Asn Ala Asp Tyr Ser Tyr Ser Val Trp Lys Gly Gly Ser Ala
1045 1050 1055

Ser Thr Trp Leu Thr Ala Phe Ala Leu Arg Val Leu Gly Gln
1060 1065 1070

Val Asn Lys Tyr Val Glu Gln Asn Gln Asn Ser Ile Cys Asn
1075 1080

Ser Leu Leu Trp Leu Val Glu Asn Tyr Gln Leu Asp Asn Gly
1085 1090 1095

Samsung Bioepis Exhibit No. 1002
Page 8681

```
Ser Phe Lys Glu Asn Ser Gln Tyr Gln Pro Ile Lys Leu Gln
    1100                1105                1110

Gly Thr Leu Pro Val Glu Ala Arg Glu Asn Ser Leu Tyr Leu
        1115                1120                1125

Thr Ala Phe Thr Val Ile Gly Ile Arg Lys Ala Phe Asp Ile
            1130                1135                1140

Cys Pro Leu Val Lys Ile Asp Thr Ala Leu Ile Lys Ala Asp
                1145                1150

Asn Phe Leu Leu Glu Asn Thr Leu Pro Ala Gln Ser Thr Phe
1155                1160                1165

Thr Leu Ala Ile Ser Ala Tyr Ala Leu Ser Leu Gly Asp Lys
    1170                1175                1180

Thr His Pro Gln Phe Arg Ser Ile Val Ser Ala Leu Lys Arg
        1185                1190                1195

Glu Ala Leu Val Lys Gly Asn Pro Pro Ile Tyr Arg Phe Trp
            1200                1205                1210

Lys Asp Asn Leu Gln His Lys Asp Ser Ser Val Pro Asn Thr
                1215                1220

Gly Thr Ala Arg Met Val Glu Thr Thr Ala Tyr Ala Leu Leu
1225                1230                1235

Thr Ser Leu Asn Leu Lys Asp Ile Asn Tyr Val Asn Pro Val
    1240                1245                1250

Ile Lys Trp Leu Ser Glu Glu Gln Arg Tyr Gly Gly Gly Phe
        1255                1260                1265
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Samsung Bioepis Exhibit No. 1002
Page 8682

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Tyr Ser Thr Gln Asp Thr Ile Asn Ala Ile Glu Gly Leu Thr
            1270                1275                1280

Glu Tyr Ser Leu Leu Val Lys Gln Leu Arg Leu Ser Met Asp
                1285                1290

Ile Asp Val Ser Tyr Lys His Lys Gly Ala Leu His Asn Tyr
1295                1300                1305

Lys Met Thr Asp Lys Asn Phe Leu Gly Arg Pro Val Glu Val
    1310                1315                1320

Leu Leu Asn Asp Asp Leu Ile Val Ser Thr Gly Phe Gly Ser
        1325                1330                1335

Gly Leu Ala Thr Val His Val Thr Thr Val Val His Lys Thr
            1340                1345                1350

Ser Thr Ser Glu Glu Val Cys Ser Phe Tyr Leu Lys Ile Asp
                1355                1360

Thr Gln Asp Ile Glu Ala Ser His Tyr Arg Gly Tyr Gly Asn
1365                1370                1375

Ser Asp Tyr Lys Arg Ile Val Ala Cys Ala Ser Tyr Lys Pro
    1380                1385                1390

Ser Arg Glu Glu Ser Ser Ser Gly Ser Ser His Ala Val Met
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Asp Ile Ser Leu Pro Thr Gly Ile Ser Ala Asn Glu Glu Asp
            1410                1415                1420

Leu Lys Ala Leu Val Glu Gly Val Asp Gln Leu Phe Thr Asp
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Tyr Gln Ile Lys Asp Gly His Val Ile Leu Gln Leu Asn Ser
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Ile Pro Ser Ser Asp Phe Leu Cys Val Arg Phe Arg Ile Phe
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Val Tyr Glu Tyr His Arg Pro Asp Lys Gln Cys Thr Met Phe
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Tyr Ser Thr Ser Asn Ile Lys Ile Gln Lys Val Cys Glu Gly
1495 1500

Ala Ala Cys Lys Cys Val Glu Ala Asp Cys Gly Gln Met Gln
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Glu Glu Leu Asp Leu Thr Ile Ser Ala Glu Thr Arg Lys Gln
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1535 1540 1545

Ile Thr Ser Ile Thr Val Glu Asn Val Phe Val Lys Tyr Lys
1550 1555 1560

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Glu Lys Asp Ser Glu Ile Thr Phe Ile Lys Lys Val Thr Cys
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Thr Asn Ala Glu Leu Val Lys Gly Arg Gln Tyr Leu Ile Met
1590 1595 1600

Gly Lys Glu Ala Leu Gln Ile Lys Tyr Asn Phe Ser Phe Arg
1605 1610 1615

Samsung Bioepis Exhibit No. 1002
Page 8684

Tyr Ile Tyr Pro Leu Asp Ser Leu Thr Trp Ile Glu Tyr Trp
1620 1625 1630

Pro Arg Asp Thr Thr Cys Ser Ser Cys Gln Ala Phe Leu Ala
1635 1640

Asn Leu Asp Glu Phe Ala Glu Asp Ile Phe Leu Asn Gly Cys
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(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4059 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: Apex-1 Eukaryotic Expression Vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GTCATTAGTT CATAGCCCAT ATATGGAGTT CCGCGTTACA TAACTTACGG 100

TAAATGGCCC CGCCTGGCTG ACCGCCCAAC GACCCCCGCC CATTGACGTC 150

AATAATGACG TATGTTCCCA TAGTAACGCC AATAGGGACT TTCCATTGAC 200

GTCAATGGGT GGACTATTTA CGGTAAACTG CCCACTTGGC AGTACATCAA 250

GTGTATCATA TGCCAAGTAC GCCCCCTATT GACGTCAATG ACGGTAAATG 300

GCCCGCCTGG CATTATGCCC AGTACATGAC CTTATGGGAC TTTCCTACTT 350

GGCAGTACAT CTACGTATTA GTCATCGCTA TTACCATGGT GATGCGGTTT 400

Samsung Bioepis Exhibit No. 1002
Page 8685

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Samsung Bioepis Exhibit No. 1002
Page 8686

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Samsung Bioepis Exhibit No. 1002
Page 8687

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Samsung Bioepis Exhibit No. 1002
Page 8688

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TGCCACCTG 4059

Samsung Bioepis Exhibit No. 1002
Page 8689

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 8540 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: Apex-3P Eukaryotic Expression Vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGACCAATA CAAAACAAAA GCGCCCCTCG TACCAGCGAA GAAGGGGCAG 50

AGATGCCGTA GTCAGGTTTA GTTCGTCCGG CGGCGGGGGA TCTGTATGGT 100

GCACTCTCAG TACAATCTGC TCTGATGCCG CATAGTTAAG CCAGTATCTG 150

CTCCCTGCTT GTGTGTTGGA GGTCGCTGAG TAGTGCGCGA GCAAAATTTA 200

AGCTACAACA AGGCAAGGCT TGACCGACAA TTGCATGAAG AATCTGCTTA 250

GGGTTAGGCG TTTTGCGCTG CTTCGCGATG TACGGGCCAG ATATACGCGT 300

TGACATTGAT TATTGACTAG TTATTAATAG TAATCAATTA CGGGGTCATT 350

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ACGTATGTTC CCATAGTAAC GCCAATAGGG ACTTTCCATT GACGTCAATG 500

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Samsung Bioepis Exhibit No. 1002
Page 8690

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Samsung Bioepis Exhibit No. 1002
Page 8691

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Samsung Bioepis Exhibit No. 1002
Page 8692

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Samsung Bioepis Exhibit No. 1002
Page 8693

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Samsung Bioepis Exhibit No. 1002
Page 8694

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Samsung Bioepis Exhibit No. 1002
Page 8695

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Samsung Bioepis Exhibit No. 1002
Page 8696

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Samsung Bioepis Exhibit No. 1002
Page 8697

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ATTGGCGTGG AGCCCCGTTT AATTTTCGGG GGTGTTAGAG ACAACCAGTG 7450

GAGTCCGCTG CTGTCGGCGT CCACTCTCTT TCCCCTTGTT ACAAATAGAG 7500

TGTAACAACA TGGTTCACCT GTCTTGGTCC CTGCCTGGGA CACATCTTAA 7550

TAACCCCAGT ATCATATTGC ACTAGGATTA TGTGTTGCCC ATAGCCATAA 7600

ATTCGTGTGA GATGGACATC CAGTCTTTAC GGCTTGTCCC CACCCCATGG 7650

ATTTCTATTG TTAAAGATAT TCAGAATGTT TCATTCCTAC ACTAGTATTT 7700

ATTGCCCAAG GGGTTTGTGA GGGTTATATT GGTGTCATAG CACAATGCCA 7750

CCACTGAACC CCCCGTCCAA ATTTTATTCT GGGGGCGTCA CCTGAAACCT 7800

TGTTTTCGAG CACCTCACAT ACACCTTACT GTTCACAACT CAGCAGTTAT 7850

TCTATTAGCT AAACGAAGGA GAATGAAGAA GCAGGCGAAG ATTCAGGAGA 7900

GTTCACTGCC CGCTCCTTGA TCTTCAGCCA CTGCCCTTGT GACTAAAATG 7950

GTTCACTACC CTCGTGGAAT CCTGACCCCA TGTAAATAAA ACCGTGACAG 8000

CTCATGGGGT GGGAGATATC GCTGTTCCTT AGGACCCTTT TACTAACCCT 8050

AATTCGATAG CATATGCTTC CCGTTGGGTA ACATATGCTA TTGAATTAGG 8100

GTTAGTCTGG ATAGTATATA CTACTACCCG GGAAGCATAT GCTACCCGTT 8150

TAGGGTTAAC AAGGGGGCCT TATAAACACT ATTGCTAATG CCCTCTTGAG 8200

GGTCCGCTTA TCGGTAGCTA CACAGGCCCC TCTGATTGAC GTTGGTGTAG 8250

Samsung Bioepis Exhibit No. 1002
Page 8698

CCTCCCGTAG TCTTCCTGGG CCCCTGGGAG GTACATGTCC CCCAGCATTG 8300

GTGTAAGAGC TTCAGCCAAG AGTTACACAT AAAGGCAATG TTGTGTTGCA 8350

GTCCACAGAC TGCAAAGTCT GCTCCAGGAT GAAAGCCACT CAGTGTTGGC 8400

AAATGTGCAC ATCCATTTAT AAGGATGTCA ACTACAGTCA GAGAACCCCT 8450

TTGTGTTTGG TCCCCCCCCG TGTCACATGT GGAACAGGGC CCAGTTGGCA 8500

AGTTGTACCA ACCAACTGAA GGGATTACAT GCACTGCCCC 8540

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 bases

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: Oligonucleotide primer UDEC690

(iii) HYPOTHETICAL: No

(iv) ANTI-SENSE: No

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGCCTGCAGG ACATCCAGAT GACTCAGTCT 30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 bases

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Single

(D) TOPOLOGY: Linear

(ii) MOLECULE TYPE: Other nucleic acid

Samsung Bioepis Exhibit No. 1002
Page 8699

(A) DESCRIPTION: Oligonucleotide primer UDEC395
(iii) HYPOTHETICAL: No
(iv) ANTI-SENSE: Yes

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CCCAAGCTTA CTGGATGGTG GGAAGATGGA 30

(2) INFORMATION FOR SEQ ID NO:7:
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 747 base pairs
(B) TYPE: Nucleic Acid
(C) STRANDEDNESS: Double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: Other nucleic acid
(A) DESCRIPTION:5G1.1M1 scFv (murine)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ATG GCC GAC ATC CAG ATG ACT CAG TCT CCA 30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
1 5 10

GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT 60
Ala Ser Leu Ser Ala Ser Val Gly Glu Thr
15 20

GTC ACC ATC ACA TGT GGA GCA AGT GAG AAT 90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
25 30

ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG 120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
35 40

Samsung Bioepis Exhibit No. 1002
Page 8700

```
AAA CAG GGA AAA TCT CCT CAG CTC CTG ATC 150
Lys Gln Gly Lys Ser Pro Gln Leu Leu Ile
                45                  50

TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG 180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Met
                55                  60

TCA TCG AGG TTC AGT GGC AGT GGA TCT GGT 210
Ser Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG 240
Arg Gln Tyr Tyr Leu Lys Ile Ser Ser Leu
                75                  80

CAT CCT GAC GAT GTT GCA ACG TAT TAC TGT 270
His Pro Asp Asp Val Ala Thr Tyr Tyr Cys
                85                  90

CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC 300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                  100

GGT GCT GGG ACC AAG TTG GAG CTG AAA CGG 330
Gly Ala Gly Thr Lys Leu Glu Leu Lys Arg
                105                 110

ACC GGA GGT GGC GGG TCG GGT GGC GGG GGA 360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCG GGT GGC GGA GGG TCG CAG GTT CAG CTG 390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130
```


Samsung Bioepis Exhibit No. 1002
Page 8701

```
CAG CAG TCT GGA GCC GAG CTG ATG AAG CCT 420
Gln Gln Ser Gly Ala Glu Leu Met Lys Pro
                135                 140

GGG GCC TCA GTG AAG ATG TCC TGC AAG GCT 450
Gly Ala Ser Val Lys Met Ser Cys Lys Ala
                145                 150

ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA 480
Thr Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAG TGG ATA AAG CAG AGG CCT GGA CAT GGC 510
Gln Trp Ile Lys Gln Arg Pro Gly His Gly
                165                 170

CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA 540
Leu Glu Trp Ile Gly Glu Ile Leu Pro Gly
                175                 180

AGT GGT TCT ACT GAG TAC ACT GAG AAC TTC 570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA 600
Lys Asp Lys Ala Ala Phe Thr Ala Asp Thr
                195                 200

TCC TCC AAC ACA GCC TAC ATG CAA CTC AGC 630
Ser Ser Asn Thr Ala Tyr Met Gln Leu Ser
                205                 210

AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT 660
Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr
                215                 220
```


Samsung Bioepis Exhibit No. 1002
Page 8702

```
TAC TGT GCA AGA TAT TTC TTC GGT AGT AGC 690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA 720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Ala
                235                 240

GGG ACC ACG GTC ACC GTC TCC TCA TGA     747
Gly Thr Thr Val Thr Val Ser Ser
                245
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 747 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1 scFv CB (humanized)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG 30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
 1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG 60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC 90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30
```


Samsung Bioepis Exhibit No. 1002
Page 8703

```
ATC TAT GGC GCG CTG AAC TGG TAT CAA CGT 120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Arg
                35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT 150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC 180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA 210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG 240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT 270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC 300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                 100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT 330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
               105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA 360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
               115                 120
```


Samsung Bioepis Exhibit No. 1002
Page 8704

```
TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG 390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA 420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT 450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT 480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC 510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170

CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC 540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT ACC GAA AAT TTT 570
Ser Gly Ser Thr Glu Tyr Thr Glu Asn Phe
                185                 190

AAA GAC CGT GTT ACT ATG ACG CGT GAC ACT 600
Lys Asp Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC 630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210
```


Samsung Bioepis Exhibit No. 1002
Page 8705

```
AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT 660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC 690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA 720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA     747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 726 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1M1 VL HuK (chimeric light chain)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC 30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC 60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10
```


Samsung Bioepis Exhibit No. 1002
Page 8706

```
CAT TCA GGT CAT AGC CTG CAG GAC ATC CAG 90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                  1               5

ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA 120
Met Thr Gln Ser Pro Ala Ser Leu Ser Ala
                10                  15

TCT GTG GGA GAA ACT GTC ACC ATC ACA TGT 150
Ser Val Gly Glu Thr Val Thr Ile Thr Cys
                20                  25

GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA 180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAT TGG TAT CAG CGG AAA CAG GGA AAA TCT 210
Asn Trp Tyr Gln Arg Lys Gln Gly Lys Ser
                40                  45

CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC 240
Pro Gln Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55

TTG GCA GAT GGC ATG TCA TCG AGG TTC AGT 270
Leu Ala Asp Gly Met Ser Ser Arg Phe Ser
                60                  65

GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC 300
Gly Ser Gly Ser Gly Arg Gln Tyr Tyr Leu
                70                  75

AAG ATC AGT AGC CTG CAT CCT GAC GAT GTT 330
Lys Ile Ser Ser Leu His Pro Asp Asp Val
                80                  85
```


Samsung Bioepis Exhibit No. 1002
Page 8707

```
GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT 360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                 90                  95

ACT CCT CTC ACG TTC GGT GCT GGG ACC AAG 390
Thr Pro Leu Thr Phe Gly Ala Gly Thr Lys
                100                 105

TTG GAG CTG AAA CGA ACT GTG GCT GCA CCA 420
Leu Glu Leu Lys Arg Thr Val Ala Ala Pro
                110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG 450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG 480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG 510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC 540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155

CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC 570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC 600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175
```


Samsung Bioepis Exhibit No. 1002
Page 8708

```
AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA 630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC 660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG 690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG 720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                                 726
Cys
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 750 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1M1 VH +HuG1 (chimeric Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ATG AAA TGG AGC TGG GTT ATT CTC TTC CTC 30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG 60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                  1
```


Samsung Bioepis Exhibit No. 1002
Page 8709

```
GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG 90
Val Gln Leu Gln Gln Ser Gly Ala Glu Leu
             5                  10

ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC 120
Met Lys Pro Gly Ala Ser Val Lys Met Ser
            15                  20

TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC 150
Cys Lys Ala Thr Gly Tyr Ile Phe Ser Asn
            25                  30

TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT 180
Tyr Trp Ile Gln Trp Ile Lys Gln Arg Pro
            35                  40

GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT 210
Gly His Gly Leu Glu Trp Ile Gly Glu Ile
            45                  50

TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT 240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
            55                  60

GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT 270
Glu Asn Phe Lys Asp Lys Ala Ala Phe Thr
            65                  70

GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG 300
Ala Asp Thr Ser Ser Asn Thr Ala Tyr Met
            75                  80

CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT 330
Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser
            85                  90
```


Samsung Bioepis Exhibit No. 1002
Page 8710

```
GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC 360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                  100

GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC 390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC 420
Trp Gly Ala Gly Thr Thr Val Thr Val Ser
            115                 120

TCA GCC TCC ACC AAG GGC CCA TCG GTC TTC 450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TGC TCC AAG AGC ACC TCT 480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC 510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG 540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC 570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC 600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180
```

```
TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG 630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG 660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC 690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG 720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA 750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 750 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1 VH + IGHRL (Humanized Fd)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC 30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
            -15                 -10
```


Samsung Bioepis Exhibit No. 1002
Page 8712

```
CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA 60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC 90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
            5                  10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC 120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
            15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT 150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
            25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC 180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
            35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC 210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
            45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC 240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
            55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT 270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
            65                  70

GCG GAC ACT TCG ACT AGT ACA GCC TAC ATG 300
Ala Asp Thr Ser Thr Ser Thr Ala Tyr Met
            75                  80
```


Samsung Bioepis Exhibit No. 1002
Page 8713

2189015

```
GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG 330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT 360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT 390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG 420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC 450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT 480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC 510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG 540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160

TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC 570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170
```

```
GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC 600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG 630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG 660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC 690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG 720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA 750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 750 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1 VH + IGHRLC (Humanized Fd)

Samsung Bioepis Exhibit No. 1002
Page 8715

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC 30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA 60
Leu Ser Val Thr Ala Gly Val His Ser Gln
               -5                   1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC 90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
            5                   10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC 120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
            15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT 150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
            25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC 180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
            35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC 210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
            45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT ACC 240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Thr
            55                  60

GAA AAT TTT AAA GAC CGT GTT ACT ATG ACG 270
Glu Asn Phe Lys Asp Arg Val Thr Met Thr
            65                  70
```


Samsung Bioepis Exhibit No. 1002
Page 8716

```
CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG 300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
             75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG 330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT 360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT 390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG 420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC 450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT 480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC 510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG 540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160
```

```
TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC 570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC 600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG 630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG 660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC 690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG 720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA 750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 726 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1 VL +KLV56 (Humanized light chain)

Samsung Bioepis Exhibit No. 1002
Page 8718

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC 30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC 60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG 90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1               5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC 120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC 150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG 180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT 210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC 240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT 270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                60                  65
```


Samsung Bioepis Exhibit No. 1002
Page 8719

```
GGA TCC GGC TCC GGA ACG GAT TAC ACT CTG 300
Gly Ser Gly Ser Gly Thr Asp Tyr Thr Leu
                 70                  75

ACC ATC AGC AGT CTG CAA CCT GAG GAC TTC 330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                 80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT 360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                 90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG 390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
                100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA 420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
                110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG 450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
                120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG 480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
                130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG 510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
                140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC 540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
                150                 155
```

```
CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC 570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC 600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA 630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC 660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG 690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG 720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                             726
Cys
```

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 726 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1 VL +KLV56B (Humanized light chain)

Samsung Bioepis Exhibit No. 1002
Page 8721

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
ATG GGA ATC CAA GGA GGG TCT GTC CTG TTC 30
Met Gly Ile Gln Gly Gly Ser Val Leu Phe
-25                 -20

GGG CTG CTG CTC GTC CTG GCT GTC TTC TGC 60
Gly Leu Leu Leu Val Leu Ala Val Phe Cys
-15                 -10

CAT TCA GGT CAT AGC CTG CAG GAT ATC CAG 90
His Ser Gly His Ser Leu Gln Asp Ile Gln
-5                   1                   5

ATG ACC CAG TCC CCG TCC TCC CTG TCC GCC 120
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala
                   10                  15

TCT GTG GGC GAT AGG GTC ACC ATC ACC TGC 150
Ser Val Gly Asp Arg Val Thr Ile Thr Cys
                   20                  25

GGC GCC AGC GAA AAC ATC TAT GGC GCG CTG 180
Gly Ala Ser Glu Asn Ile Tyr Gly Ala Leu
                   30                  35

AAC TGG TAT CAA CGT AAA CCT GGG AAA GCT 210
Asn Trp Tyr Gln Arg Lys Pro Gly Lys Ala
                   40                  45

CCG AAG CTT CTG ATT TAC GGT GCG ACG AAC 240
Pro Lys Leu Leu Ile Tyr Gly Ala Thr Asn
                   50                  55

CTG GCA GAT GGA GTC CCT TCT CGC TTC TCT 270
Leu Ala Asp Gly Val Pro Ser Arg Phe Ser
                   60                  65
```


Samsung Bioepis Exhibit No. 1002
Page 8722

```
GGA TCC GGC TCC GGA ACG GAT TTC ACT CTG 300
Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu
                70                  75

ACC ATC AGC AGT CTG CAG CCT GAA GAC TTC 330
Thr Ile Ser Ser Leu Gln Pro Glu Asp Phe
                80                  85

GCT ACG TAT TAC TGT CAG AAC GTT TTA AAT 360
Ala Thr Tyr Tyr Cys Gln Asn Val Leu Asn
                90                  95

ACT CCG TTG ACT TTC GGA CAG GGT ACC AAG 390
Thr Pro Leu Thr Phe Gly Gln Gly Thr Lys
               100                 105

GTG GAA ATA AAA CGA ACT GTG GCT GCA CCA 420
Val Glu Ile Lys Arg Thr Val Ala Ala Pro
               110                 115

TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG 450
Ser Val Phe Ile Phe Pro Pro Ser Asp Glu
               120                 125

CAG TTG AAA TCT GGA ACT GCC TCT GTT GTG 480
Gln Leu Lys Ser Gly Thr Ala Ser Val Val
               130                 135

TGC CTG CTG AAT AAC TTC TAT CCC AGA GAG 510
Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu
               140                 145

GCC AAA GTA CAG TGG AAG GTG GAT AAC GCC 540
Ala Lys Val Gln Trp Lys Val Asp Asn Ala
               150                 155
```


Samsung Bioepis Exhibit No. 1002
Page 8723

```
CTC CAA TCG GGT AAC TCC CAG GAG AGT GTC 570
Leu Gln Ser Gly Asn Ser Gln Glu Ser Val
                160                 165

ACA GAG CAG GAC AGC AAG GAC AGC ACC TAC 600
Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr
                170                 175

AGC CTC AGC AGC ACC CTG ACG CTG AGC AAA 630
Ser Leu Ser Ser Thr Leu Thr Leu Ser Lys
                180                 185

GCA GAC TAC GAG AAA CAC AAA GTC TAC GCC 660
Ala Asp Tyr Glu Lys His Lys Val Tyr Ala
                190                 195

TGC GAA GTC ACC CAT CAG GGC CTG AGC TCG 690
Cys Glu Val Thr His Gln Gly Leu Ser Ser
                200                 205

CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG 720
Pro Val Thr Lys Ser Phe Asn Arg Gly Glu
                210                 215

TGT TAG                             726
Cys
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 711 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1 VL + O12 (Humanized light chain)

Samsung Bioepis Exhibit No. 1002
Page 8724

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
ATG GAC ATG AGG GTC CCC GCT CAG CTC CTG 30
Met Asp Met Arg Val Pro Ala Gln Leu Leu
            -20                 -15

GGG CTC CTG CTA CTC TGG CTC CGA GGT GCC 60
Gly Leu Leu Leu Leu Trp Leu Arg Gly Ala
            -10                 -5

AGA TGT GAT ATC CAG ATG ACC CAG TCC CCG 90
Arg Cys Asp Ile Gln Met Thr Gln Ser Pro
         1               5

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG 120
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
     10              15

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC 150
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
     20                  25

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG 180
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
     30                  35

AAA CCC GGG AAA GCT CCG AAG CTT CTG ATT 210
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
     40                  45

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC 240
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
     50                  55

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA 270
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
     60                  65
```


Samsung Bioepis Exhibit No. 1002
Page 8725

```
ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG 300
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
     70                  75

CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT 330
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
     80                  85

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC 360
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
     90                  95

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGA 390
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
    100                 105

ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC 420
Thr Val Ala Ala Pro Ser Val Phe Ile Phe
    110                 115

CCG CCA TCT GAT GAG CAG TTG AAA TCT GGA 450
Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly
    120                 125

ACT GCC TCT GTT GTG TGC CTG CTG AAT AAC 480
Thr Ala Ser Val Val Cys Leu Leu Asn Asn
    130                 135

TTC TAT CCC AGA GAG GCC AAA GTA CAG TGG 510
Phe Tyr Pro Arg Glu Ala Lys Val Gln Trp
    140                 145

AAG GTG GAT AAC GCC CTC CAA TCG GGT AAC 540
Lys Val Asp Asn Ala Leu Gln Ser Gly Asn
    150                 155
```


Samsung Bioepis Exhibit No. 1002
Page 8726

```
TCC CAG GAG AGT GTC ACA GAG CAG GAC AGC 570
Ser Gln Glu Ser Val Thr Glu Gln Asp Ser
    160                 165

AAG GAC AGC ACC TAC AGC CTC AGC AGC ACC 600
Lys Asp Ser Thr Tyr Ser Leu Ser Ser Thr
    170                 175

CTG ACG CTG AGC AAA GCA GAC TAC GAG AAA 630
Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys
    180                 185

CAC AAA GTC TAC GCC TGC GAA GTC ACC CAT 660
His Lys Val Tyr Ala Cys Glu Val Thr His
    190                 195

CAG GGC CTG AGC TCG CCC GTC ACA AAG AGC 690
Gln Gly Leu Ser Ser Pro Val Thr Lys Ser
    200                 205

TTC AAC AGG GGA GAG TGT TAG             711
Phe Asn Arg Gly Glu Cys
    210
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 750 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION:5G1.1 VH + IGHRLD (Humanized Fd)

Samsung Bioepis Exhibit No. 1002
Page 8727

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
ATG AAG TGG AGC TGG GTT ATT CTC TTC CTC 30
Met Lys Trp Ser Trp Val Ile Leu Phe Leu
               -15                 -10

CTG TCA GTA ACT GCC GGC GTC CAC TCC CAA 60
Leu Ser Val Thr Ala Gly Val His Ser Gln
                -5                  1

GTC CAA CTG GTG CAA TCC GGC GCC GAG GTC 90
Val Gln Leu Val Gln Ser Gly Ala Glu Val
             5                  10

AAG AAG CCA GGG GCC TCA GTC AAA GTG TCC 120
Lys Lys Pro Gly Ala Ser Val Lys Val Ser
             15                  20

TGT AAA GCT AGC GGC TAT ATT TTT TCT AAT 150
Cys Lys Ala Ser Gly Tyr Ile Phe Ser Asn
             25                  30

TAT TGG ATT CAA TGG GTG CGT CAG GCC CCC 180
Tyr Trp Ile Gln Trp Val Arg Gln Ala Pro
             35                  40

GGG CAG GGC CTG GAA TGG ATG GGT GAG ATC 210
Gly Gln Gly Leu Glu Trp Met Gly Glu Ile
             45                  50

TTA CCG GGC TCT GGT AGC ACC GAA TAT GCC 240
Leu Pro Gly Ser Gly Ser Thr Glu Tyr Ala
             55                  60

CAA AAA TTC CAG GGC CGT GTT ACT ATG ACT 270
Gln Lys Phe Gln Gly Arg Val Thr Met Thr
             65                  70
```

```
CGT GAC ACT TCG ACT AGT ACA GTA TAC ATG 300
Arg Asp Thr Ser Thr Ser Thr Val Tyr Met
             75                  80

GAG CTC TCC AGC CTG CGA TCG GAG GAC ACG 330
Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr
             85                  90

GCC GTC TAT TAT TGC GCG CGT TAT TTT TTT 360
Ala Val Tyr Tyr Cys Ala Arg Tyr Phe Phe
             95                 100

GGT TCT AGC CCG AAT TGG TAT TTT GAT GTT 390
Gly Ser Ser Pro Asn Trp Tyr Phe Asp Val
            105                 110

TGG GGT CAA GGA ACC CTG GTC ACT GTC TCG 420
Trp Gly Gln Gly Thr Leu Val Thr Val Ser
            115                 120

AGC GCC TCC ACC AAG GGC CCA TCG GTC TTC 450
Ser Ala Ser Thr Lys Gly Pro Ser Val Phe
            125                 130

CCC CTG GCG CCC TCC TCC AAG AGC ACC TCT 480
Pro Leu Ala Pro Ser Ser Lys Ser Thr Ser
            135                 140

GGG GGC ACA GCG GCC CTG GGC TGC CTG GTC 510
Gly Gly Thr Ala Ala Leu Gly Cys Leu Val
            145                 150

AAG GAC TAC TTC CCC GAA CCG GTG ACG GTG 540
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val
            155                 160
```


Samsung Bioepis Exhibit No. 1002
Page 8729

```
TCG TGG AAC TCA GGC GCC CTG ACC AGC GGC 570
Ser Trp Asn Ser Gly Ala Leu Thr Ser Gly
            165                 170

GTG CAC ACC TTC CCG GCT GTC CTA CAG TCC 600
Val His Thr Phe Pro Ala Val Leu Gln Ser
            175                 180

TCA GGA CTC TAC TCC CTC AGC AGC GTG GTG 630
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val
            185                 190

ACC GTG CCC TCC AGC AGC TTG GGC ACC CAG 660
Thr Val Pro Ser Ser Ser Leu Gly Thr Gln
            195                 200

ACC TAC ATC TGC AAC GTG AAT CAC AAG CCC 690
Thr Tyr Ile Cys Asn Val Asn His Lys Pro
            205                 210

AGC AAC ACC AAG GTG GAC AAG AAA GTT GAG 720
Ser Asn Thr Lys Val Asp Lys Lys Val Glu
            215                 220

CCC AAA TCT TGT GAC AAA ACT CAC ACA TAA 750
Pro Lys Ser Cys Asp Lys Thr His Thr
            225                 230
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 747 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

Samsung Bioepis Exhibit No. 1002
Page 8730

(A) DESCRIPTION:5G1.1 scFv.D012
(Humanized scFv)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
ATG GCC GAT ATC CAG ATG ACC CAG TCC CCG 30
Met Ala Asp Ile Gln Met Thr Gln Ser Pro
 1               5                  10

TCC TCC CTG TCC GCC TCT GTG GGC GAT AGG 60
Ser Ser Leu Ser Ala Ser Val Gly Asp Arg
                15                  20

GTC ACC ATC ACC TGC GGC GCC AGC GAA AAC 90
Val Thr Ile Thr Cys Gly Ala Ser Glu Asn
                25                  30

ATC TAT GGC GCG CTG AAC TGG TAT CAA CAG 120
Ile Tyr Gly Ala Leu Asn Trp Tyr Gln Gln
                35                  40

AAA CCT GGG AAA GCT CCG AAG CTT CTG ATT 150
Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile
                45                  50

TAC GGT GCG ACG AAC CTG GCA GAT GGA GTC 180
Tyr Gly Ala Thr Asn Leu Ala Asp Gly Val
                55                  60

CCT TCT CGC TTC TCT GGA TCC GGC TCC GGA 210
Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
                65                  70

ACG GAT TTC ACT CTG ACC ATC AGC AGT CTG 240
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu
                75                  80
```


Samsung Bioepis Exhibit No. 1002
Page 8731

```
CAG CCT GAA GAC TTC GCT ACG TAT TAC TGT 270
Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys
                85                  90

CAG AAC GTT TTA AAT ACT CCG TTG ACT TTC 300
Gln Asn Val Leu Asn Thr Pro Leu Thr Phe
                95                  100

GGA CAG GGT ACC AAG GTG GAA ATA AAA CGT 330
Gly Gln Gly Thr Lys Val Glu Ile Lys Arg
                105                 110

ACT GGC GGT GGT GGT TCT GGT GGC GGT GGA 360
Thr Gly Gly Gly Gly Ser Gly Gly Gly Gly
                115                 120

TCT GGT GGT GGC GGT TCT CAA GTC CAA CTG 390
Ser Gly Gly Gly Gly Ser Gln Val Gln Leu
                125                 130

GTG CAA TCC GGC GCC GAG GTC AAG AAG CCA 420
Val Gln Ser Gly Ala Glu Val Lys Lys Pro
                135                 140

GGG GCC TCA GTC AAA GTG TCC TGT AAA GCT 450
Gly Ala Ser Val Lys Val Ser Cys Lys Ala
                145                 150

AGC GGC TAT ATT TTT TCT AAT TAT TGG ATT 480
Ser Gly Tyr Ile Phe Ser Asn Tyr Trp Ile
                155                 160

CAA TGG GTG CGT CAG GCC CCC GGG CAG GGC 510
Gln Trp Val Arg Gln Ala Pro Gly Gln Gly
                165                 170
```


Samsung Bioepis Exhibit No. 1002
Page 8732

```
CTG GAA TGG ATG GGT GAG ATC TTA CCG GGC 540
Leu Glu Trp Met Gly Glu Ile Leu Pro Gly
                175                 180

TCT GGT AGC ACC GAA TAT GCC CAA AAA TTC 570
Ser Gly Ser Thr Glu Tyr Ala Gln Lys Phe
                185                 190

CAG GGC CGT GTT ACT ATG ACG CGT GAC ACT 600
Gln Gly Arg Val Thr Met Thr Arg Asp Thr
                195                 200

TCG ACT AGT ACA GTA TAC ATG GAG CTC TCC 630
Ser Thr Ser Thr Val Tyr Met Glu Leu Ser
                205                 210

AGC CTG CGA TCG GAG GAC ACG GCC GTC TAT 660
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr
                215                 220

TAT TGC GCG CGT TAT TTT TTT GGT TCT AGC 690
Tyr Cys Ala Arg Tyr Phe Phe Gly Ser Ser
                225                 230

CCG AAT TGG TAT TTT GAT GTT TGG GGT CAA 720
Pro Asn Trp Tyr Phe Asp Val Trp Gly Gln
                235                 240

GGA ACC CTG GTC ACT GTC TCG AGC TGA    747
Gly Thr Leu Val Thr Val Ser Ser
                245
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 5248 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

Samsung Bioepis Exhibit No. 1002
Page 8733

(D) TOPOLOGY: Circular

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: pET Trc S05/NI
prokaryotic expression vector

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TGGCGAATGG GACGCGCCCT GTAGCGGCGC ATTAAGCGCG GCGGGTGTGG 50

TGGTTACGCG CAGCGTGACC GCTACACTTG CCAGCGCCCT AGCGCCCGCT 100

CCTTTCGCTT TCTTCCCTTC CTTTCTCGCC ACGTTCGCCG GCTTTCCCCG 150

TCAAGCTCTA AATCGGGGGC TCCCTTTAGG GTTCCGATTT AGTGCTTTAC 200

GGCACCTCGA CCCCAAAAAA CTTGATTAGG GTGATGGTTC ACGTAGTGGG 250

CCATCGCCCT GATAGACGGT TTTTCGCCCT TTGACGTTGG AGTCCACGTT 300

CTTTAATAGT GGACTCTTGT TCCAAACTGG AACAACACTC AACCCTATCT 350

CGGTCTATTC TTTTGATTTA TAAGGGATTT TGCCGATTTC GGCCTATTGG 400

TTAAAAAATG AGCTGATTTA ACAAAAATTT AACGCGAATT TTAACAAAAT 450

ATTAACGTTT ACAATTTCAG GTGGCACTTT TCGGGGAAAT GTGCGCGGAA 500

CCCCTATTTG TTTATTTTTC TAAATACATT CAAATATGTA TCCGCTCATG 550

AGACAATAAC CCTGATAAAT GCTTCAATAA TATTGAAAAA GGAAGAGTAT 600

GAGTATTCAA CATTTCCGTG TCGCCCTTAT TCCCTTTTTT GCGGCATTTT 650

GCCTTCCTGT TTTTGCTCAC CCAGAAACGC TGGTGAAAGT AAAAGATGCT 700

GAAGATCAGT TGGGTGCACG AGTGGGTTAC ATCGAACTGG ATCTCAACAG 750

Samsung Bioepis Exhibit No. 1002
Page 8734

CGGTAAGATC CTTGAGAGTT TTCGCCCCGA AGAACGTTTT CCAATGATGA 800

GCACTTTTAA AGTTCTGCTA TGTGGCGCGG TATTATCCCG TATTGACGCC 850

GGGCAAGAGC AACTCGGTCG CCGCATACAC TATTCTCAGA ATGACTTGGT 900

TGAGTACTCA CCAGTCACAG AAAAGCATCT TACGGATGGC ATGACAGTAA 950

GAGAATTATG CAGTGCTGCC ATAACCATGA GTGATAACAC TGCGGCCAAC 1000

TTACTTCTGA CAACGATCGG AGGACCGAAG GAGCTAACCG CTTTTTTGCA 1050

CAACATGGGG GATCATGTAA CTCGCCTTGA TCGTTGGGAA CCGGAGCTGA 1100

ATGAAGCCAT ACCAAACGAC GAGCGTGACA CCACGATGCC TGCAGCAATG 1150

GCAACAACGT TGCGCAAACT ATTAACTGGC GAACTACTTA CTCTAGCTTC 1200

CCGGCAACAA TTAATAGACT GGATGGAGGC GGATAAAGTT GCAGGACCAC 1250

TTCTGCGCTC GGCCCTTCCG GCTGGCTGGT TTATTGCTGA TAAATCTGGA 1300

GCCGGTGAGC GTGGGTCTCG CGGTATCATT GCAGCACTGG GGCCAGATGG 1350

TAAGCCCTCC CGTATCGTAG TTATCTACAC GACGGGGAGT CAGGCAACTA 1400

TGGATGAACG AAATAGACAG ATCGCTGAGA TAGGTGCCTC ACTGATTAAG 1450

CATTGGTAAC TGTCAGACCA AGTTTACTCA TATATACTTT AGATTGATTT 1500

AAAACTTCAT TTTTAATTTA AAAGGATCTA GGTGAAGATC CTTTTTGATA 1550

ATCTCATGAC CAAAATCCCT TAACGTGAGT TTTCGTTCCA CTGAGCGTCA 1600

GACCCCGTAG AAAAGATCAA AGGATCTTCT TGAGATCCTT TTTTTCTGCG 1650

CGTAATCTGC TGCTTGCAAA CAAAAAAACC ACCGCTACCA GCGGTGGTTT 1700

Samsung Bioepis Exhibit No. 1002
Page 8735

GTTTGCCGGA TCAAGAGCTA CCAACTCTTT TTCCGAAGGT AACTGGCTTC 1750

AGCAGAGCGC AGATACCAAA TACTGTCCTT CTAGTGTAGC CGTAGTTAGG 1800

CCACCACTTC AAGAACTCTG TAGCACCGCC TACATACCTC GCTCTGCTAA 1850

TCCTGTTACC AGTGGCTGCT GCCAGTGGCG ATAAGTCGTG TCTTACCGGG 1900

TTGGACTCAA GACGATAGTT ACCGGATAAG GCGCAGCGGT CGGGCTGAAC 1950

GGGGGGTTCG TGCACACAGC CCAGCTTGGA GCGAACGACC TACACCGAAC 2000

TGAGATACCT ACAGCGTGAG CTATGAGAAA GCGCCACGCT TCCCGAAGGG 2050

AGAAAGGCGG ACAGGTATCC GGTAAGCGGC AGGGTCGGAA CAGGAGAGCG 2100

CACGAGGGAG CTTCCAGGGG GAAACGCCTG GTATCTTTAT AGTCCTGTCG 2150

GGTTTCGCCA CCTCTGACTT GAGCGTCGAT TTTTGTGATG CTCGTCAGGG 2200

GGGCGGAGCC TATGGAAAAA CGCCAGCAAC GCGGCCTTTT TACGGTTCCT 2250

GGCCTTTTGC TGGCCTTTTG CTCACATGTT CTTTCCTGCG TTATCCCCTG 2300

ATTCTGTGGA TAACCGTATT ACCGCCTTTG AGTGAGCTGA TACCGCTCGC 2350

CGCAGCCGAA CGACCGAGCG CAGCGAGTCA GTGAGCGAGG AAGCGGAAGA 2400

GCGCCTGATG CGGTATTTTC TCCTTACGCA TCTGTGCGGT ATTTCACACC 2450

GCATATATGG TGCACTCTCA GTACAATCTG CTCTGATGCC GCATAGTTAA 2500

GCCAGTATAC ACTCCGCTAT CGCTACGTGA CTGGGTCATG GCTGCGCCCC 2550

GACACCCGCC AACACCCGCT GACGCGCCCT GACGGGCTTG TCTGCTCCCG 2600

GCATCCGCTT ACAGACAAGC TGTGACCGTC TCCGGGAGCT GCATGTGTCA 2650

Samsung Bioepis Exhibit No. 1002
Page 8736

GAGGTTTTCA CCGTCATCAC CGAAACGCGC GAGGCAGCTG CGGTAAAGCT 2700

CATCAGCGTG GTCGTGAAGC GATTCACAGA TGTCTGCCTG TTCATCCGCG 2750

TCCAGCTCGT TGAGTTTCTC CAGAAGCGTT AATGTCTGGC TTCTGATAAA 2800

GCGGGCCATG TTAAGGGCGG TTTTTTCCTG TTTGGTCACT GATGCCTCCG 2850

TGTAAGGGGG ATTTCTGTTC ATGGGGGTAA TGATACCGAT GAAACGAGAG 2900

AGGATGCTCA CGATACGGGT TACTGATGAT GAACATGCCC GGTTACTGGA 2950

ACGTTGTGAG GGTAAACAAC TGGCGGTATG GATGCGGCGG GACCAGAGAA 3000

AAATCACTCA GGGTCAATGC CAGCGCTTCG TTAATACAGA TGTAGGTGTT 3050

CCACAGGGTA GCCAGCAGCA TCCTGCGATG CAGATCCGGA ACATAATGGT 3100

GCAGGGCGCT GACTTCCGCG TTTCCAGACT TTACGAAACA CGGAAACCGA 3150

AGACCATTCA TGTTGTTGCT CAGGTCGCAG ACGTTTTGCA GCAGCAGTCG 3200

CTTCACGTTC GCTCGCGTAT CGGTGATTCA TTCTGCTAAC CAGTAAGGCA 3250

ACCCCGCCAG CCTAGCCGGG TCCTCAACGA CAGGAGCACG ATCATGCGCA 3300

CCCGTGGGGC CGCCATGCCG GCGATAATGG CCTGCTTCTC GCCGAAACGT 3350

TTGGTGGCGG GACCAGTGAC GAAGGCTTGA GCGAGGGCGT GCAAGATTCC 3400

GAATACCGCA AGCGACAGGC CGATCATCGT CGCGCTCCAG CGAAAGCGGT 3450

CCTCGCCGAA AATGACCCAG AGCGCTGCCG GCACCTGTCC TACGAGTTGC 3500

ATGATAAAGA AGACAGTCAT AAGTGCGGCG ACGATAGTCA TGCCCCGCGC 3550

CCACCGGAAG GAGCTGACTG GGTTGAAGGC TCTCAAGGGC ATCGGTCGAG 3600

Samsung Bioepis Exhibit No. 1002
Page 8737

ATCCCGGTGC CTAATGAGTG AGCTAACTTA CATTAATTGC GTTGCGCTCA 3650

CTGCCCGCTT TCCAGTCGGG AAACCTGTCG TGCCAGCTGC ATTAATGAAT 3700

CGGCCAACGC GCGGGGAGAG GCGGTTTGCG TATTGGGCGC CAGGGTGGTT 3750

TTTCTTTTCA CCAGTGAGAC GGGCAACAGC TGATTGCCCT TCACCGCCTG 3800

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AATTTGATTG CGAGTGAGAT ATTTATGCCA GCCAGCCAGA CGCAGACGCG 4150

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AATAATACTG TTGATGGGTG TCTGGTCAGA GACATCAAGA AATAACGCCG 4300

GAACATTAGT GCAGGCAGCT TCCACAGCAA TGGCATCCTG GTCATCCAGC 4350

GGATAGTTAA TGATCAGCCC ACTGACGCGT TGCGCGAGAA GATTGTGCAC 4400

CGCCGCTTTA CAGGCTTCGA CGCCGCTTCG TTCTACCATC GACACCACCA 4450

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GACGGCGCGT GCAGGGCCAG ACTGGAGGTG GCAACGCCAA TCAGCAACGA 4550

Samsung Bioepis Exhibit No. 1002
Page 8738

CTGTTTGCCC GCCAGTTGTT GTGCCACGCG GTTGGGAATG TAATTCAGCT 4600

CCGCCATCGC CGCTTCCACT TTTTCCCGCG TTTTCGCAGA AACGTGGCTG 4650

GCCTGGTTCA CCACGCGGGA AACGGTCTGA TAAGAGACAC CGGCATACTC 4700

TGCGACATCG TATAACGTTA CTGGTTTCAC ATTCACCACC CTGAATTGAC 4750

TCTCTTCCGG GCGCTATCAT GCCATACCGC GAAAGGTTTT GCGCCATTCG 4800

ATGGTGTCCG GGATCTCGAC GCTCTCCCTT ATGCGACTCC TGCATTAGGA 4850

AGCAGCCCAG TAGTAGGTTG AGGCCGTTGA GCACCGCCGC CGCAAGGAAT 4900

GGTGCATGCG GTACCAGCTG TTGACAATTA ATCATCCGGC TCGTATAATA 4950

GTACTGTGTG GAATTGTGAG CGCTCACAAT TCCACACATC TAGAAATAAT 5000

TTTGTTTAAC TTTAAGAAGG AGATATACCA TGGAGATCTG GATCCATCGA 5050

TGAATTCGAG CTCCGTCGAC AAGCTTGCGG CCGCACTCGA GCACCACCAC 5100

CACCACCACT GAGATCCGGC TGCTAACAAA GCCCGAAAGG AAGCTGAGTT 5150

GGCTGCTGCC ACCGCTGAGC AATAACTAGC ATAACCCCTT GGGGCCTCTA 5200

AACGGGTCTT GAGGGGTTTT TTGCTGAAAG GAGGAACTAT ATCCGGAT 5248

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 813 base pairs

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(A) DESCRIPTION: N19/8 scFv (His Tagged)

Samsung Bioepis Exhibit No. 1002
Page 8739

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

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ATG GCC AAT ATT GTG CTG ACC CAA TCT CCA 30
Met Ala Asn Ile Val Leu Thr Gln Ser Pro
 1               5                  10

GCT TCT TTG GCT GTG TCT CTA GGG CAG AGG 60
Ala Ser Leu Ala Val Ser Leu Gly Gln Arg
                15                  20

GCC ACC ATA TCC TGC AGA GCC AGT GAA AGT 120
Ala Thr Ile Ser Cys Arg Ala Ser Glu Ser
                25                  30

GTT GAT AGT TAT GAC AAT AGT TTT ATG CAC 150
Val Asp Ser Tyr Asp Asn Ser Phe Met His
                35                  40

TGG TAC CAG CAG AAA CCA GGA CAG CCA CCC 180
Trp Tyr Gln Gln Lys Pro Gly Gln Pro Pro
                45                  50

AAA CTC CTC ATC TTT CTT GCA TCC AAC CTA 210
Lys Leu Leu Ile Phe Leu Ala Ser Asn Leu
                55                  60

GAA TCT GGG GTC CCT GCC AGG TTC AGT GGC 240
Glu Ser Gly Val Pro Ala Arg Phe Ser Gly
                65                  70

AGT GGG TCT AGG ACA GAC TTC ACC CTC ACC 270
Ser Gly Ser Arg Thr Asp Phe Thr Leu Thr
                75                  80

ATT GAT CCT GTG GAG GCT GAT GAT GCT GCA 300
Ile Asp Pro Val Glu Ala Asp Asp Ala Ala
                85                  90
```

```
ACC TAT TAC TGT CAG CAA AAT AAT GAG GTT 330
Thr Tyr Tyr Cys Gln Gln Asn Asn Glu Val
 95                 100

CCG AAC ACG TTC GGA GGG GGG ACC AAG CTG 360
Pro Asn Thr Phe Gly Gly Gly Thr Lys Leu
 105                110

GAA ATA AAA CGG ACC GGA GGT GGC GGG TCG 390
Glu Ile Lys Arg Thr Gly Gly Gly Gly Ser
 115                120

GGT GGC GGG GGA TCG GGT GGC GGA GGG TCG 420
Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
 125                130

GAC GTC AAG CTC GTG GAG TCT GGG GGA GAC 450
Asp Val Lys Leu Val Glu Ser Gly Gly Asp
 135                140

TTA GTG AAG CTT GGA GGG TCC CTG AAA CTC 480
Leu Val Lys Leu Gly Gly Ser Leu Lys Leu
 145                150

TCC TGT GCA GCC TCT GGA TTC ACC TTC AGT 510
Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser
 155                160

AGC TAT TAT ATG TCT TGG GTT CGC CAG ATT 540
Ser Tyr Tyr Met Ser Trp Val Arg Gln Ile
 165                170

TCA GAG AAG AGG CTG GAG TTG GTC GCA GCC 570
Ser Glu Lys Arg Leu Glu Leu Val Ala Ala
 175                180
```


Samsung Bioepis Exhibit No. 1002
Page 8741

```
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Ile Asn Ser Asn Gly Asp Ser Thr Tyr Tyr
                185                 190

CCA GAC ACT GTG AAG GGC CGA TTC ACC ATC 630
Pro Asp Thr Val Lys Gly Arg Phe Thr Ile
                195                 200

TCC AGA GAC AAT GCC AAG AGC ACC CTG GAT 660
Ser Arg Asp Asn Ala Lys Ser Thr Leu Asp
                205                 210

CTG CAA ATG AGC AGT CTG AAG TCT GAG GAC 690
Leu Gln Met Ser Ser Leu Lys Ser Glu Asp
                215                 220

ACA GCC TTG TAT TTC TGT GTA AGA GAG ACT 720
Thr Ala Leu Tyr Phe Cys Val Arg Glu Thr
                225                 230

TAT TAC TAC GGG ATT AGT CCC GTC TTC GAT 750
Tyr Tyr Tyr Gly Ile Ser Pro Val Phe Asp
                235                 240

GTC TGG GGC ACA GGG ACC ACG GTC ACC GTC 780
Val Trp Gly Thr Gly Thr Thr Val Thr Val
                245                 250

TCC TCA CTC GAG CAC CAC CAC CAC CAC CAC 810
Ser Ser Leu Glu His His His His His His
                255                 260

TGA                                     813
```


Samsung Bioepis Exhibit No. 1002
Page 8742

WO 95/29697 PCT/US95/05688

2189015

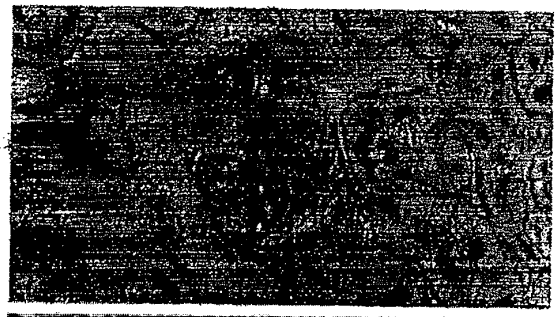


FIG. 1A



FIG. 1B

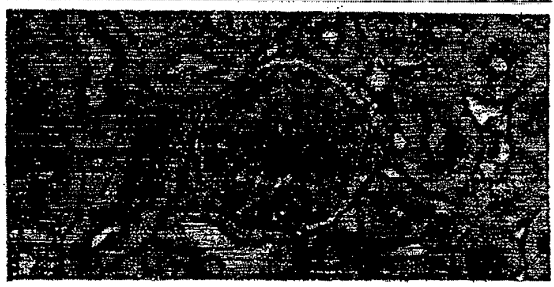


FIG. 1C

Samsung Bioepis Exhibit No. 1002
Page 8743



FIG. 2A



FIG. 2B

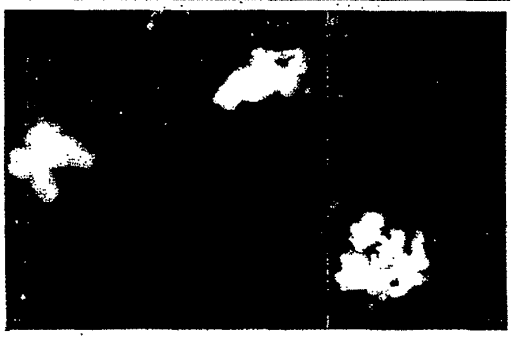


FIG. 2C

Samsung Bioepis Exhibit No. 1002
Page 8744

2189015

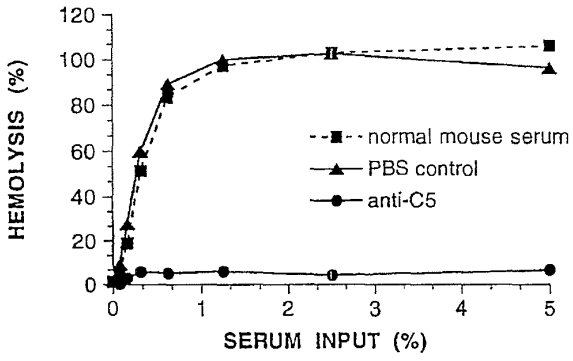


FIG. 3

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Page 8745

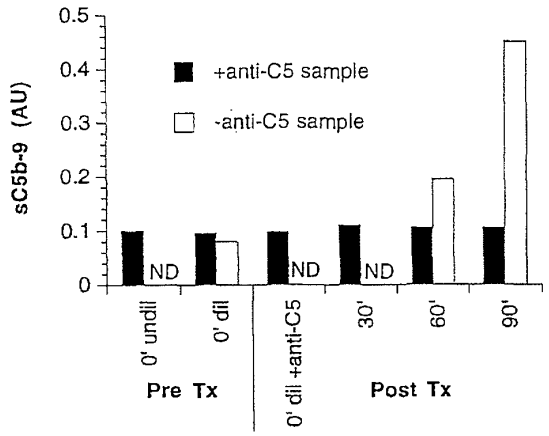


FIG. 4

Samsung Bioepis Exhibit No. 1002
Page 8746

SUBSTITUTE SHEET (RULE 26)

FIG. 5A

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WO 95/19697 PCT/US95/00688

Samsung Bioepis Exhibit No. 1002
Page 8747

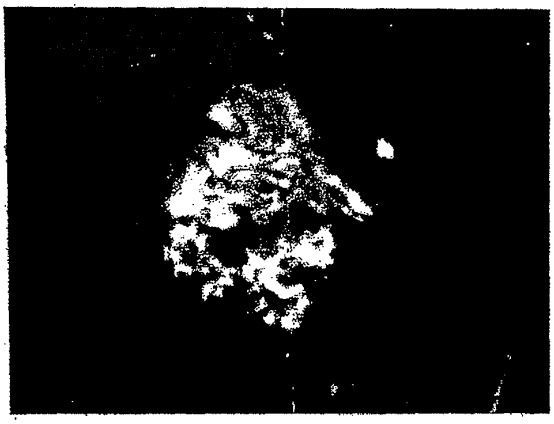


FIG.5B

Samsung Bioepis Exhibit No. 1002
Page 8748



FIG. 5C

Samsung Bioepis Exhibit No. 1002
Page 8749

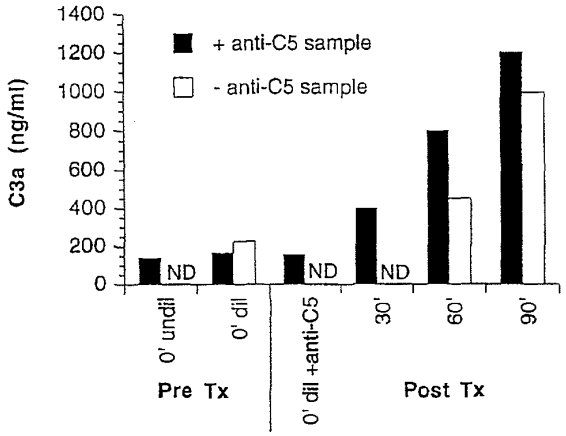


FIG. 6

Samsung Bioepis Exhibit No. 1002
Page 8750

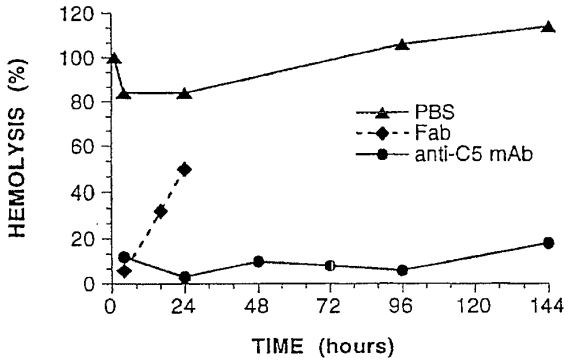


FIG. 7A

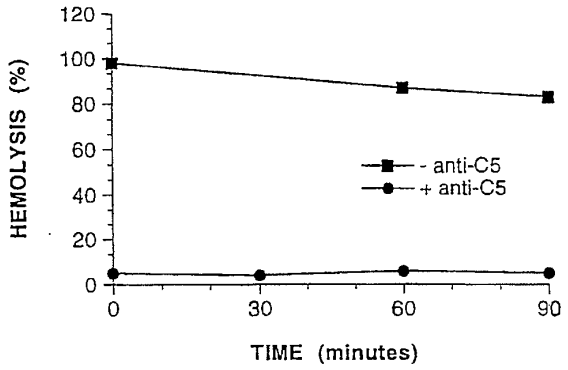


FIG. 7B

Samsung Bioepis Exhibit No. 1002
Page 8751

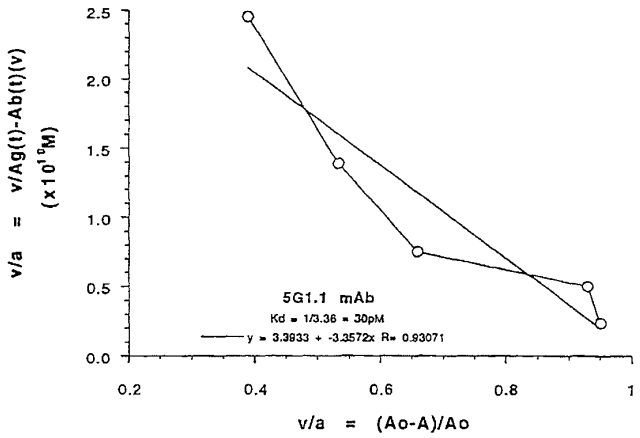


Fig. 8

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Page 8752

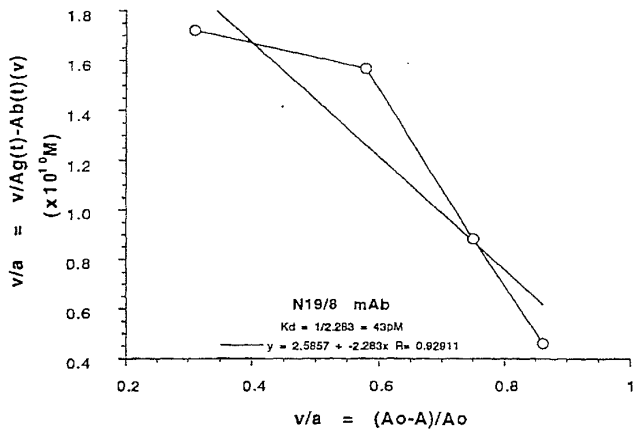


Fig. 9

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Page 8753

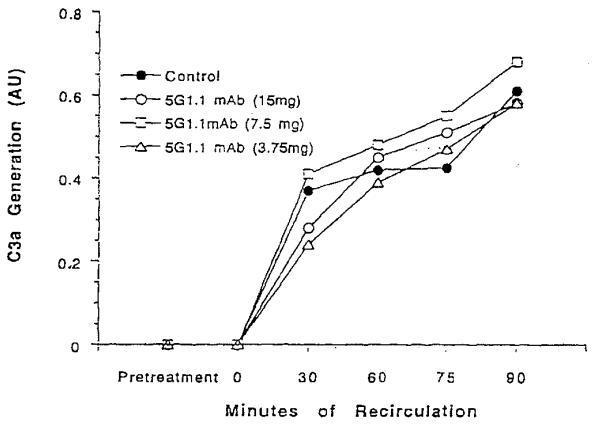


Fig. 10

Samsung Bioepis Exhibit No. 1002
Page 8754

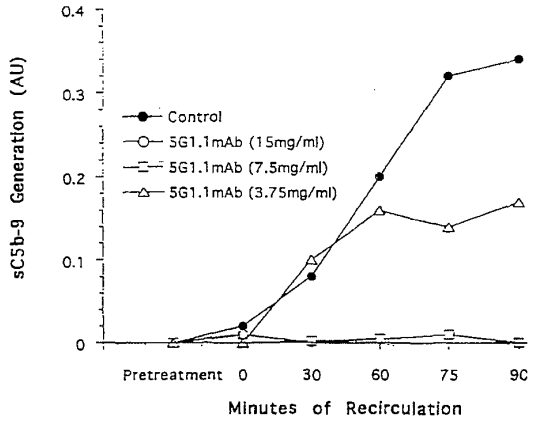


Fig. 11

Samsung Bioepis Exhibit No. 1002
Page 8755

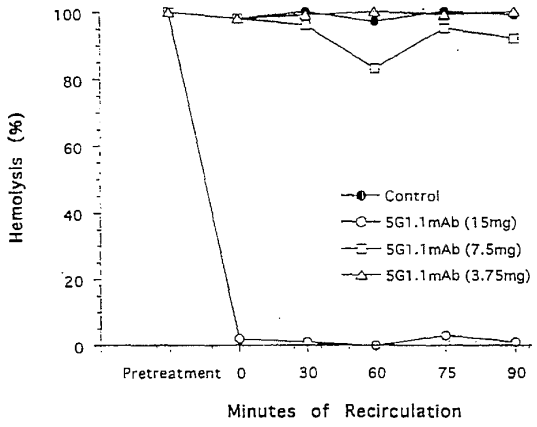


Fig. 12

Samsung Bioepis Exhibit No. 1002
Page 8756

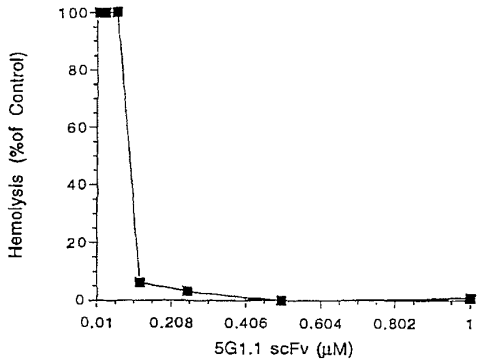


Fig. 13

Samsung Bioepis Exhibit No. 1002
Page 8757

2189015

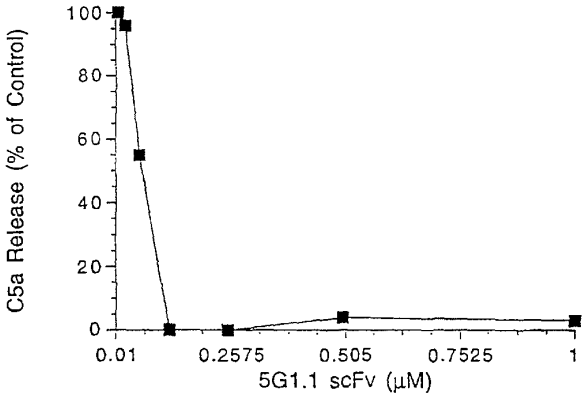


Fig. 14

Samsung Bioepis Exhibit No. 1002
Page 8758

2189015

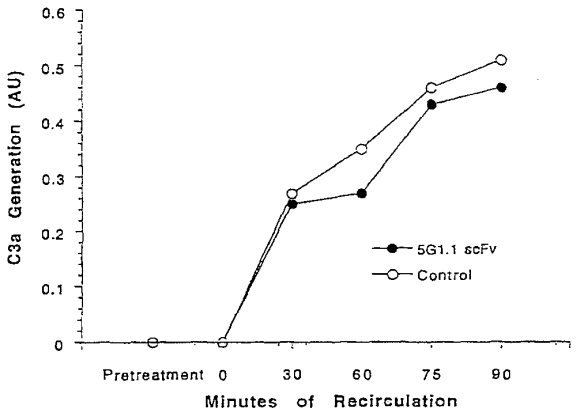


Fig. 15

Samsung Bioepis Exhibit No. 1002
Page 8759

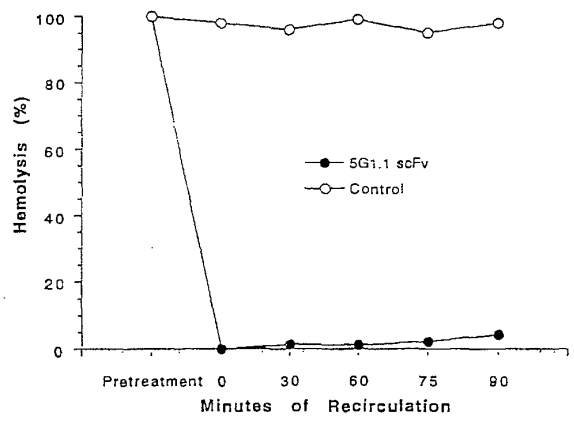


Fig. 16

Samsung Bioepis Exhibit No. 1002
Page 8760

2189015

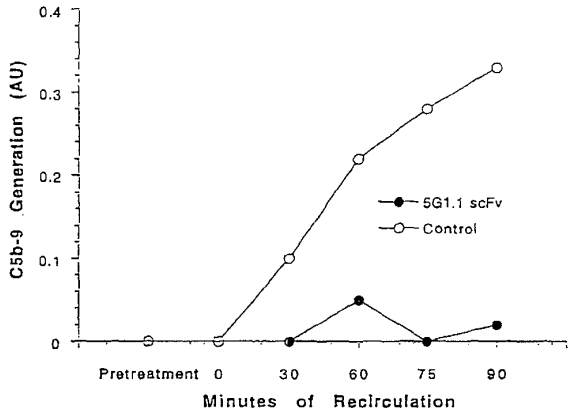


Fig. 17

Samsung Bioepis Exhibit No. 1002
Page 8761

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1                                                                  10                                                                  20
GAC ATC CAG ATG ACT CAG TCT CCA GCT TCA CTG TCT GCA TCT GTG GGA GAA ACT GTC ACC
D   I   Q   M   T   Q   S   P   A   S   L   S   A   S   V   G   E   T   V   T

                24                                 30                  34                                 40
ATC ACA TGT GGA GCA AGT GAG AAT ATT TAC GGT GCT TTA AAT TGG TAT CAG CGG AAA CAG
I   T   C   G   A   S   E   N   I   Y   G   A   L   N   W   Y   Q   R   K   Q
                    CDR-L1

                                                   50                                 56                  60
GGA AAA TCT CCT CAG CTC CTG ATC TAT GGT GCA ACC AAC TTG GCA GAT GGC ATG TCA TCG
G   K   S   P   Q   L   L   I   Y   G   A   T   N   L   A   D   G   M   S   S
                                        CDR-L2

                                                   70                                                      80
AGG TTC AGT GGC AGT GGA TCT GGT AGA CAG TAT TAT CTC AAG ATC AGT AGC CTG CAT CCT
R   F   S   G   S   G   S   G   R   Q   Y   Y   L   K   I   S   S   L   H   P

                                                                  89                                  97      100
GAC GAT GTT GCA ACG TAT TAC TGT CAA AAT GTG TTA AAT ACT CCT CTC ACG TTC GGT GCT
D   D   V   A   T   Y   Y   C   Q   N   V   L   N   T   P   L   T   F   G   A
                                                    CDR-L3

GGG ACC AAG TTG GAG CTG AAA
G   T   K   L   E   L   K
```

Fig. 18

```
-19                                                                  -1 +1
atg aaa tgg agc tgg gtt att ctc ttC CTC CTG TCA GTA ACT GCA GGT GTC CAC TCC CAG
 M  K  W  S  W  V  I  L  F  L  L  S  V  T  A  G  V  H  S  Q

                                10                                  20
GTT CAG CTG CAG CAG TCT GGA GCT GAG CTG ATG AAG CCT GGG GCC TCA GTG AAG ATG TCC
 V  Q  L  Q  Q  S  G  A  E  L  M  K  P  G  A  S  V  K  M  S

                26              30                  35              40
TGC AAG GCT ACT GGC TAC ATA TTC AGT AAC TAC TGG ATA CAG TGG ATA AAG CAG AGG CCT
 C  K  A  T  G  Y  I  F  S  N  Y  W  I  Q  W  I  K  Q  R  P
                                CDR-H1
                                50        52a                       60
GGA CAT GGC CTT GAG TGG ATT GGT GAG ATT TTA CCT GGA AGT GGT TCT ACT GAG TAC ACT
 G  H  G  L  E  W  I  G  E  I  L  P  G  S  G  S  T  E  Y  T
                                         CDR-H2
                65              70                                  80
GAG AAC TTC AAG GAC AAG GCC GCA TTC ACT GCA GAT ACA TCC TCC AAC ACA GCC TAC ATG
 E  N  F  K  D  K  A  A  F  T  A  D  T  S  S  N  T  A  Y  M

       82a 82b 82c                      90                  95
CAA CTC AGC AGC CTG ACA TCA GAG GAC TCT GCC GTC TAT TAC TGT GCA AGA TAT TTC TTC
 Q  L  S  S  L  T  S  E  D  S  A  V  Y  Y  C  A  R  Y  F  F

       100 a  b  c  d  e    102                          110
GGT AGT AGC CCC AAC TGG TAC TTC GAT GTC TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC
 G  S  S  P  N  W  Y  F  D  V  W  G  A  G  T  T  V  T  V  S
       CDR-H3

TCA
 S
```

Fig. 19

BE

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Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2198706 C 2008/07/08

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CANADIAN PATENT

(13) C

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(87) Date publication PCT/PCT Publication Date: 1996/03/28
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(85) Entrée phase nationale/National Entry: 1997/02/27
(86) N° demande PCT/PCT Application No.: US 1995/012404
(87) N° publication PCT/PCT Publication No.: 1996/009043
(30) Priorité/Priority: 1994/09/23 (US08/311,489)

(51) Cl.Int./Int.Cl. *A61K 39/395* (2006.01), *A61P 19/02* (2006.01), *A61P 29/00* (2006.01), *C07K 16/18* (2006.01)

(72) Inventeurs/Inventors:
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MATIS, LOUIS, US

(73) Propriétaire/Owner:
ALEXION PHARMACEUTICALS, INC., US

(74) Agent: MCFADDEN, FINCHAM

(54) Titre : PROCEDES DE TRAITEMENT DE L'ARTHRITE
(54) Title: METHODS FOR THE TREATMENT OF INFLAMMATORY JOINT DISEASE

(57) Abrégé/Abstract:
The use of compounds that block complement component C5 or its active fragments C5a and/or C5b (such compounds collectively referred to as "C5 blockers") to treat established joint inflammation (arthritis) is disclosed. Administration of such C5 blockers has been found to: 1) arrest and/or reduce inflammation in joints which are already inflamed, and 2) inhibit the spread of inflammation to unaffected joints.

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International Bureau

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08/311,489 23 September 1994 (23.09.94) US

(71) Applicant: ALEXION PHARMACEUTICALS, INC. [US/US]; Suite 360, 25 Science Park, New Haven, CT 06511 (US).

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(74) Agent: KLEE, Maurice, M.; 1951 Burr Street, Fairfield, CT 06430 (US).

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(54) Title: METHODS FOR THE TREATMENT OF INFLAMMATORY JOINT DISEASE

(57) Abstract

The use of compounds that block complement component C5 or its active fragments C5a and/or C5b (such compounds collectively referred to as "C5 blockers") to treat established joint inflammation (arthritis) is disclosed. Administration of such C5 blockers has been found to: 1) arrest and/or reduce inflammation in joints which are already inflamed, and 2) inhibit the spread of inflammation to unaffected joints.

Samsung Bioepis Exhibit No. 1002
Page 8765

CLAIMS:

1. Use of an effective anti-inflammatory amount of an anti-C5 monoclonal antibody for the treatment of established joint inflammation.

2. The use of claim 1, wherein the anti-C5 monoclonal antibody is in an amount sufficient to substantially inhibit a cell-lysing ability of complement present in a blood-derived fluid of a patient.

3. The use of claim 2, wherein the blood-derived fluid is serum.

4. The use of claim 1, wherein the anti-C5 monoclonal antibody is in an amount sufficient to substantially reduce a level of soluble C5b-9 present in a blood-derived fluid of a patient after activation of complement in that fluid.

5. The use of claim 4, wherein the blood-derived fluid is serum.

6. The use of claim 1, wherein the anti-C5 monoclonal antibody is in an amount sufficient to substantially reduce a level of C5a present in a blood-derived fluid of a patient after activation of complement in that fluid.

7. The use of claim 6, wherein the blood-derived fluid is serum.

8. The use of claim 1, wherein the anti-C5 monoclonal antibody is in an amount sufficient to reduce a cell-lysing ability of complement present in synovial fluid of an inflamed joint of a patient by at least 10%.

9. The use of claim 1, wherein the anti-C5 monoclonal antibody is in an amount sufficient to reduce a level of soluble C5b-9 present in synovial fluid of an inflamed joint of a patient by at least 10%.

10. The use of claim 1, wherein the anti-C5 monoclonal antibody is in an amount sufficient to reduce a level of C5a present in synovial fluid of an inflamed joint of a patient by at least 10%.

11. The use of claim 1, wherein the anti-C5 monoclonal antibody does not substantially interfere with cleavage of

Samsung Bioepis Exhibit No. 1002
Page 8766

complement component C3 in a patient's serum into C3a and C3b.

12. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises an anti-C5 monoclonal antibody which provides the agent with anti-inflammatory properties; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of joint inflammation.

13. An article of manufacture comprising packaging material and a pharmaceutical agent contained within said packaging material, wherein:

(a) said pharmaceutical agent comprises an anti-C5 monoclonal antibody which provides the agent with anti-inflammatory properties; and

(b) said packaging material comprises a label which indicates that said pharmaceutical agent is for use in the treatment of arthritis.

Samsung Bioepis Exhibit No. 1002
Page 8767

METHODS FOR THE TREATMENT OF INFLAMMATORY JOINT DISEASE

FIELD OF THE INVENTION

The present invention relates to methods for the treatment of inflammatory joint disease. In particular, the invention relates to the use of blockers of complement component C5 ("C5 blockers") as pharmaceutical agents to treat established joint inflammation.

BACKGROUND OF THE INVENTION

I. The Complement System

The complement system acts in conjunction with other immunological systems of the body to defend against intrusion of cellular and viral pathogens. There are at least 25 complement proteins, which are found as a complex collection of plasma proteins and membrane cofactors. The plasma proteins (which are also found in most other body fluids, such as lymph, bone marrow, synovial fluid, and cerebrospinal fluid) make up about 10% of the globulins in vertebrate serum. Complement components achieve their immune defensive functions by interacting in a series of intricate but precise enzymatic cleavage and membrane binding events. The resulting complement cascade leads to the production of products with opsonic, immunoregulatory, and lytic functions.

The complement cascade progresses via the classical pathway or the alternative pathway. These pathways share many components, and, while they differ in their early steps, both converge and share the same terminal complement components responsible for the destruction of target cells and viruses.

The classical complement pathway is typically initiated by antibody recognition of and binding to an

Samsung Bioepis Exhibit No. 1002
Page 8768

antigenic site on a target cell. This surface bound antibody subsequently reacts with the first component of complement, C1. The C1 thus bound undergoes a set of autocatalytic reactions that result in, inter alia, the induction of C1 proteolytic activity acting on complement components C2 and C4.

This activated C1 cleaves C2 and C4 into C2a, C2b, C4a, and C4b. The function of C2b is poorly understood. C2a and C4b combine to form the C4b,2a complex, which is an active protease known as classical C3 convertase. C4b,2a acts to cleave C3 into C3a and C3b. C3a and C4a are both relatively weak anaphylatoxins that may induce degranulation of mast cells, resulting in the release of histamine and other mediators of inflammation.

C3b has multiple functions. As opsonin, it binds to bacteria, viruses and other cells and particles and tags them for removal from the circulation. C3b can also form a complex with C4b,C2a to produce C4b,2a,3b, or classical C5 convertase, which cleaves C5 into C5a (another anaphylatoxin), and C5b. Alternative C5 convertase is C3b, Bb, C3b and performs the same function. C5b combines with C6 yielding C5b,6, and this complex combines with C7 to form the ternary complex C5b,6,7. The C5b,6,7 complex binds C8 at the surface of a cell membrane. Upon binding of C9, the complete membrane attack complex (MAC) is formed (C5b-9) which mediates the lysis of foreign cells, microorganisms, and viruses.

Further discussions of the classical complement pathway, as well as a detailed description of the alternative pathway of complement activation, can be found in numerous publications including, for example, Roitt, et al., 1988, and Muller-Eberhard, 1988.

II. Joint Inflammation

A variety of common medical disorders have as a common element the inflammation of the patient's joints. In the United States alone, millions of patients suffer from such joint inflammation. Afflicted individuals are

Samsung Bioepis Exhibit No. 1002
Page 8769

frequently disabled, and the costs of medical care for patients suffering from such disorders are significant. While numerous means are available for treatment of joint inflammation, and new treatments continue to become available, none of these is as safe and effective as could be desired, and there has thus been a long felt need for new approaches and better methods to control joint inflammation.

Clinically, joint inflammation is associated with joint stiffness, pain, weakness, and sometimes joint fatigue. Uniformly, the joint is tender and swollen, and often erythematous. Diagnosis of the inflammatory nature of the joint disease is frequently based upon this typical clinical presentation as well as upon radiographic examination and aspiration and examination of synovial joint fluid. Examination of joint fluid of an inflamed joint generally reveals elevation of various markers of inflammation, such as, leukocytes (including neutrophils), antibodies, cytokines, cell adhesion molecules, and complement activation products (De Clerck et al., 1989; Heinz et al., 1989; Moffat et al., 1989; Peake et al., 1989; Brodeur et al., 1991; Firestein et al., 1991; Matsubara et al., 1991; Olsen et al., 1991; Oleesky et al., 1991; Jose et al., 1990; Zvaifler, 1968; Zvaifler, 1969a; Zvaifler, 1969b; Zvaifler, 1974; Ward and Zvaifler, 1971; Ward, 1975; Moxley and Ruddy, 1985; Mollnes et al., 1986; Auda et al., 1990; Olmez et al., 1991; Kahle et al., 1992; Koch et al., 1994; Thorbecke et al., 1992; Saura et al., 1992; Feldman et al., 1990; Feldman et al., 1991; Fong et al., 1994; Harigi et al., 1993; Morgan et al., 1988; Shingu et al., 1994; Abbink et al., 1992; and Corvetta et al., 1992). Radiographic examination of affected joints generally reveals soft tissue swelling and/or erosive changes.

Joint inflammation is associated with a group of diseases that are referred to medically as arthridities (types of arthritis). The term "arthritis" is used

Samsung Bioepis Exhibit No. 1002
Page 8770

medically to generally describe diseases of the joints. The term, however, is also used to describe certain medical conditions, of which rheumatoid arthritis (RA) is the primary example, that consist of a multiplicity of different pathologic manifestations, including, but by no means limited to, joint disease.

Discussions of arthritis may thus include diseases such as RA, where joint disorders are only one facet of the varied pathologies associated with the disease. The present invention is directed specifically to the joint disorder aspects of these diseases. The methods of the invention, however, may also have beneficial effects on non-joint-associated pathologies. For example, use of the methods of the invention to treat established joint inflammation associated with RA, psoriasis, lupus, and other disorders may also provide therapeutic benefits impacting on some of the other pathologic manifestations of these multifaceted disease states, such as vascular inflammation and nephritis (see, for example, Wurzner, et al., Complement Inflamm., 8:328-340, 1991, U.S. Patent No. 5,853,722 and U.S. Patent No. 6,074,642, and Sims, et al., U.S. Patent No. 5,135,916).

It should be noted that the present invention is not concerned with all types of joint disorders, but only those involving inflammation. Thus, for example, the invention is applicable to the treatment of late stage osteoarthritis (OA), which is an inflammatory joint disease, but generally not to early stage OA, which does not typically have a significant inflammatory component.

Detailed discussions of the arthridities can be found in numerous medical texts, including Arnett, 1992, Cecil Textbook of Medicine, Wyngaarden et al. (eds.), W.B. Saunders Company, Philadelphia, Chapter 258, pp. 1508-1515; Lipsky, 1994, Harrison's Principles of Internal Medicine, 13th Ed., Isselbacher et al. (eds), McGraw-Hill, Inc., New York, Chapter 285, pp. 1648-1655;

Samsung Bioepis Exhibit No. 1002
Page 8771

and McCarty and Koopman, 1993. Arthritis and Allied Conditions, 12th Ed. Lea and Febiger, Philadelphia. As discussed in detail in these and other texts, and reviewed below, joint inflammation is associated with numerous local and systemic disease processes.

Factors Associated with Joint Inflammation

Joint inflammation is a complex process involving, among other things, activation of both cellular and humoral immune responses.

Cellular immune responses include infiltration by white blood cells, predominantly neutrophils (also referred to as polymorphonuclear cells or PMNs). Mononuclear white blood cell infiltrates are also common in many inflamed joints. Infiltrating mononuclear cells, including T lymphocytes (as well as cells resident within the joint such as synovial cells, fibroblasts, and endothelial cells) are activated and contribute to the production of multiple inflammatory cytokines including TNF-α, IL-1, IFN-γ, IL-2, IL-6, IL-8, GM-CSF, PDGF and FGF, these latter two being capable of stimulating synovial cell proliferation.

All of these cytokines are thought to play a role in inducing the production of numerous other inflammatory factors as well as various other mediators of tissue degradation. These factors and mediators of degradation include products of arachidonic acid metabolism (that are active in various intracellular signal transduction pathways), reactive oxygen intermediates, and degradative enzymes such as collagenase, stromelysin, and other neutral proteases, all of which can further contribute to the inflammatory response and to tissue destruction.

Cellular infiltration into the synovium is enhanced by the upregulation of cell adhesion molecules such as selectins, LFA-3, and members of the ICAM family of Ig-like cell adhesion molecules on cells within the joint. These adhesion molecules promote the infiltration of activated white blood cells into the affected joint by

Samsung Bioepis Exhibit No. 1002
Page 8772

stimulating leukocyte (including lymphocyte) adhesion, migration, and activation.

The humoral immune system also contributes to joint inflammation. Antibodies are produced within inflamed joints in such diseases as RA, JRA and OA (see below) and generate localized immune complexes that can activate the complement system. As discussed above in greater detail, activated complement components can have cytolytic, cell activating, anaphylatoxic, and chemotactic effects.

These multifactorial inflammatory responses lead to cartilage destruction and bone erosion that ultimately result in the joint deformity seen in patients with chronic joint inflammation.

In view of the complex nature of joint inflammation, a variety of theories, many of which are conflicting, have been proposed in the art to explain the relative importance of the various factors involved. Notwithstanding extensive work, there remains a basic controversy in the art as to the relative roles of the cellular and humoral immune systems in joint inflammation, including what role complement plays in such inflammation. See, for example, Andersson and Holmdahl, 1990; Brahn and Trentham, 1989; Chiocchia et al., 1990; Chiocchia et al., 1991; Durie et al., 1993; Goldschmidt et al., 1990; Goldschmidt and Holmdahl, 1991; Holmdahl et al., 1985; Holmdahl et al., 1989; Holmdahl et al., 1990; Hom et al., 1988; Hom et al., 1992; Hom et al., 1993; Mori et al., 1992; Myers et al., 1989A; Nakajima et al., 1993; Osman et al., 1993; Peterman et al., 1993; Seki et al., 1988; Seki et al., 1992; Terato et al., 1992; Watson et al., 1987; and, in particular, the following reports relating to the complement system and/or the relative roles of T cells and complement components in joint inflammation: Andersson et al., 1991; Andersson et al., 1992; Banerjee et al., 1988B; Banerjee et al., 1989; David, 1992; Fava et al., 1993; Haqqi et al., 1989; Kakimoto et al., 1988; Maeurer et

Samsung Bioepis Exhibit No. 1002
Page 8773

al., 1992; Morgan et al., 1981; Moxley and Ruddy, 1985; Reife et al., 1991; Spinella et al., 1991; Spinella and Stuart, 1992; van Lent et al., 1992; Watson et al., 1987; Watson and Townes, 1985; and Williams et al., 1992A. To date, these wide ranging studies have not led to effective treatments for established joint inflammation based on modulation of the complement system and, in particular, based on the use of C5 blockers.

The studies of the prophylactic effects of C5 blockers reported below in Example 2 were designed to determine if C5 was an appropriate and effective target for pharmacological modulation of the humoral immune system in order to prevent joint inflammation. The surprising effectiveness of C5 blockers in preventing onset of joint inflammation led to the design and execution of the studies reported in Example 1 in which C5 blockers were used to treat established inflammation. At the outset of these experiments, it was anticipated that such treatment would have little measurable effect upon established joint inflammation, as it was supposed that C5 was more important early in the disease process when the chemotactic activity of C5a would trigger the infiltration of inflammatory cells. It was further supposed that the involvement of T cells in established disease would continue to provide significant inflammatory stimuli even in the absence of C5 activity. As shown by the results of Example 1 this expectation was incorrect in that C5 blockers were found to be surprisingly effective in arresting and/or reducing the inflammation of joints which were already inflamed, while at the same time inhibiting the spread of inflammation to unaffected joints.

Diseases Commonly Associated with Joint Inflammation

Rheumatoid arthritis (RA) and juvenile onset rheumatoid arthritis (JRA) are chronic multisystem diseases of unknown cause. RA affects approximately 1% of the population, with women affected three times more

Samsung Bioepis Exhibit No. 1002
Page 8774

commonly than men. The onset is most frequent during the fourth and fifth decades of life. RA and JRA are systemic diseases with numerous pathologic manifestations in addition to their joint inflammatory aspects. In RA, these manifestations include RA vasculitis (inflammation of the blood vessels), which can affect nearly any organ system and can cause numerous pathologic sequelae including polyneuropathy, cutaneous ulceration, and visceral infarction. Pleuropulmonary manifestations include pleuritis, interstitial fibrosis, pleuropulmonary nodules, pneumonitis, and arteritis. Other manifestations include the development of inflammatory rheumatoid nodules on a variety of periarticular structures such as extensor surfaces, as well as on pleura and meninges. Weakness and atrophy of skeletal muscle are common.

The joint inflammation aspects of RA present as persistent inflammatory synovitis, usually involving peripheral joints in a symmetric distribution. In general, the complex intraarticular inflammatory response seen in RA is of the type described above in the general discussion of joint inflammation.

Many patients with systemic lupus erythematosis (SLE) also develop joint inflammation referred to as lupus arthritis. SLE is an autoimmune disease of unknown cause in which numerous different cells, tissues, and organs are damaged by pathogenic autoantibodies and immune complexes. Clinical manifestations of SLE are numerous and include a variety of maculopapular rashes, nephritis, cerebritis, vasculitis, hematologic abnormalities including cytopenias and coagulopathies, pericarditis, myocarditis, pleurisy, gastrointestinal symptoms, and the aforementioned joint inflammation.

Osteoarthritis (OA) represents the most common joint disease of mankind, and OA of the knee is the leading cause of chronic disability in developed countries. It is manifested by pain, stiffness, and swelling of the

Samsung Bioepis Exhibit No. 1002
Page 8775

involved joints. Articular cartilage, responsible for the most critical mechanical functions of the joint, is the major target tissue of OA. The breakdown of articular cartilage in OA is mediated by various enzymes such as metalloproteinases, plasmin, and cathepsin, which are in turn stimulated by various factors that can also act as inflammatory mediators. These factors include cytokines such as interleukin-1, which is known to activate the pathogenic cartilage and synovial proteases.

The identification of above normal levels of immunoglobulins in cartilage in generalized OA and the demonstration of type II collagen-specific antibodies in some OA patients provide evidence of a role for immune activation in this disease state (see, for example, Jasin, 1989). The observation that OA rarely remains monoarticular also suggests that this disease is a systemic disorder of articular cartilage. Synovial inflammation becomes more frequent as the disease progresses. In fact, in late stage OA, histologic evidence of synovial inflammation may be as marked as that in the synovium of patients with RA-associated joint inflammation.

Psoriatic arthritis is a chronic inflammatory joint disorder that affects 5 to 8% of people with psoriasis. A significant percentage of these individuals (one-fourth) develop progressive destructive disease. Twenty-five percent of psoriasis patients with joint inflammation develop symmetric joint inflammation resembling the joint inflammation manifestations of RA, and over half of these go on to develop varying degrees of joint destruction.

Other Diseases Associated with Joint Inflammation

A variety of other systemic illnesses have joint inflammation as a prominent feature of the clinical presentation.

Peripheral joint inflammation occurs in as many as one-fifth of patients with inflammatory bowel disease.

Samsung Bioepis Exhibit No. 1002
Page 8776

2198706

The joint inflammation is acute, associated with flare-ups of the bowel disease, and is manifested by swollen, erythematous, warm, and painful joints. Synovial fluids of sufferers have an acute inflammatory exudate of mostly neutrophils, and radiographs demonstrate soft tissue swelling and effusions.

The synovitis that accompanies hepatitis B resembles serum sickness, with abrupt onset of fever and articular inflammation. There is generally a symmetric inflammation of joints including the knee, shoulder, wrist, ankles, elbow, and the joints of the hands. Immune complexes containing hepatitis B antigens are present in serum and synovium, lending support to the concept that the synovitis is immunologically mediated. Other viral diseases associated with joint inflammation include rubella, human immunodeficiency virus infection, coxsackieviral, and adenoviral infections.

An immune complex mediated joint inflammation is also associated with intestinal bypass surgery, and joint inflammation is a prominent manifestation of Whipple's disease, or intestinal lipodystrophy, where fever, edema, serositis, lymphadenopathy, uveitis, and cerebral inflammation are associated findings. Furthermore, potentially immunologically-related joint inflammation is an associated sequelum of infectious endocarditis and certain spirochetal infections, most notably infection with Borrelia burgdorferi, the causative organism of Lyme disease.

Primary Sjögrens syndrome is a chronic, slowly progressive autoimmune disease characterized by lymphocytic infiltration of the exocrine glands resulting in xerostomia and dry eyes. One-third of patients present with systemic manifestations, including vasculitis, nephritis, mononeuritis multiplex, and, most commonly, joint inflammation.

Ankylosing spondylitis (AS) is an inflammatory disorder of unknown cause that affects primarily the

Samsung Bioepis Exhibit No. 1002
Page 8777

axial skeleton, but peripheral joints are also affected. Its incidence correlates with the HLA-B27 histocompatibility haplotype, and immune-mediated mechanisms are further implicated by elevated serum levels of IgA and an inflammatory joint histology with similar characteristics to those seen in the joint inflammation aspects of RA. Thus, synovial fluid from inflammatory peripheral joints in AS is not distinctly different from that of other inflammatory joint diseases.

Reactive arthritis (ReA) refers to acute nonpurulent joint inflammation complicating an infection elsewhere in the body. Reactive arthritis is believed to be immunologically mediated. Included in this category is the constellation of clinical findings often referred to as Reiter's syndrome or Reiter's disease. In addition to joint inflammation, this syndrome affects the skin, eyes, mucous membranes, and less commonly the heart, lungs, and nervous system. Reiter's syndrome may follow enteric infections with any of several Shigella, Salmonella, Yersinia, and Campylobacter species, and genital infections with Chlamydia trachomatous. The histology of joints affected by this syndrome is similar to that seen in other types of joint inflammation. The joint inflammation is usually quite painful, and tense joint effusions are not uncommon, especially in the knee. The joint inflammation is usually asymmetric and additive, with involvement of new joints occurring over a period of a few days to several weeks.

III. Current Therapies

Current therapies for the various types of joint inflammation discussed above include the administration of anti-inflammatory drugs such as non-steroidal drugs, including aspirin, and non-specific immunosuppressive drugs, such as gold compounds, corticosteroids, penicillamine, hydroxychloroquine, methotrexate, azathioprine, alkylating agents such as cyclophosphamide, and sulfasalazine. Administration of each of these

Samsung Bioepis Exhibit No. 1002
Page 8778

2198706

agents is sometimes associated with severe side effects and toxicities. Patients receiving certain of these treatments are also exposed to the dangers of opportunistic infection and increased risk of neoplasia associated with generalized immunosuppression. In addition to the medical texts cited above, discussions of drugs used to treat established joint inflammation can be found in Goodman and Gilman's The Pharmacological Basis of Therapeutics 18th Ed., Gilman et al. (eds.) 1990, Pergamon Press, Inc., New York, Chapter 26, pp. 638-681; Physician's Desk Reference 47th Ed., 1993, Medical Economics Co., Inc., Montvale, NJ; The United States Pharmacopeia 22nd Ed., 1989, Mack Printing Co., Easton, PA; Drug Evaluations Annual 1991, 1990, American Medical Association, Milwaukee, WI; and Cash and Klippel, 1994, N. Eng. J. Med. 330, pp. 1368-1375.

In addition to pharmacologic treatments, relief of the symptoms of joint inflammation is sometimes achieved with warm or cold soaks. Surgical intervention using tendon release procedures and/or joint replacement procedures is frequently the last resort for treatment of chronic joint inflammation. Such orthopedic surgery is associated with increased infection and prostheses have limited life spans.

New therapeutic approaches currently being developed include attempts to address various elements of the cellular immune response contributing to the inflammatory cascade present in inflamed joints. These approaches include the administration of therapeutic preparations including anti-T cell and/or anti-cytokine agents (see, for example, Banerjee et al., 1988A; Cannon et al., 1990; Chiocchia et al., 1991; Elliot et al., 1993; Fava et al., 1993; Fujimori et al., 1993; Griswold et al., 1988; Hom et al., 1988; Hom et al., 1991; Hom et al., 1993; Inoue et al., 1993; Kakimoto et al., 1992; Kleinau et al., 1989; Myers et al., 1989b; Myers et al., 1993; Nagler-Anderson et al., 1986; Nishikaku and Koga, 1993; Peterman

Samsung Bioepis Exhibit No. 1002
Page 8779

et al., 1993; Piguet et al., 1992; Smith et al., 1990; Spannaus-Martin et al., 1990; Thompson et al., 1988; Trentham et al., 1993; Williams et al., 1992b; Williams et al, 1994; and Wolos et al., 1993).

SUMMARY OF THE INVENTION

In view of the foregoing, it is an object of the present invention to provide a new approach for treating established joint inflammation.

To achieve this goal, the invention provides methods involving the use of blockers of complement component C5 as pharmaceutical agents to accomplish therapeutic treatment of established joint inflammation. The C5 blockers are administered to animals, e.g., humans, having at least one inflamed joint. The blockers can be administered systemically or locally. They achieve a reduction or stabilization of the inflammation of joints that are already inflamed and inhibit the spread of inflammation to unaffected joints.

In accordance with an embodiment of the present invention there is provided use of an effective anti-inflammatory amount of an anti-C5 monoclonal antibody for the treatment of established joint inflammation.

Yet another embodiment of the present invention provides an article of manufacture comprising packaging material and a pharmaceutical agent contained within the packaging material, wherein: (a) the pharmaceutical agent comprises an anti-C5 monoclonal antibody which provides the agent with anti-inflammatory properties; and (b) the packaging material comprises a label which indicates that the pharmaceutical agent is for use in the treatment of joint inflammation.

A still further embodiment of the present invention provides an article of manufacture comprising packaging material and a pharmaceutical agent contained within the packaging material, wherein: (a) the pharmaceutical agent comprises an anti-C5 monoclonal antibody which provides the agent with anti-inflammatory properties; and (b) the packaging material

Samsung Bioepis Exhibit No. 1002
Page 8780

comprises a label which indicates that the pharmaceutical agent is for use in the treatment of arthritis.

As used herein, a "C5 blocker" is a compound that directly interacts with C5, C5a, and/or C5b, i.e., a compound that directly binds to or directly modified (i.e., by a direct chemical reaction) one of these complement components, so as to inhibit the formation of and/or physiologic function of C5a and/or C5b. Preferably, the formation and/or physiologic functions of both C5a and C5b are inhibited by the C5 blocker.

Direct interaction with C5, C5a, and/or C5b has the important advantage that other components of the complement cascade may be left intact. In particular, the opsonization functions associated with the activation of complement component C3 by a C3 convertase to yield C3a and C3b may be left intact allowing for continued clearance of foreign particles and substances from the body by the action of C3b. The most preferred C5 blockers are those which are of this type, i.e., those that do not interfere with C3b function.

Samsung Bioepis Exhibit No. 1002
Page 8781

As demonstrated by the examples presented below, in accordance with the invention, it has been surprisingly found that treatment with C5 blockers will arrest and, in many cases, at least partially reverse the disease process at an inflamed joint, while at the same time preventing progression of joint inflammation to non-affected joints. Given the prior understanding in the art regarding the role of the cellular immune system in joint inflammation (see above), one would not have expected that a C5 blocker would have such a dramatic beneficial effect on established joint inflammation.

The accompanying figures, which are incorporated in and constitute part of the specification, illustrate certain aspects of the invention, and together with the description, serve to explain the principles of the invention. It is to be understood, of course, that both the figures and the description are explanatory only and are not restrictive of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1a-c are photomicrographs of joints stained with Hematoxylin and Eosin illustrating the use of a C5 blocker to stop the progression of established joint inflammation. Figure 1a shows a paw joint from a normal mouse; Figure 1b shows an initially affected paw joint from a control treated mouse, where the joint exhibits extensive bone erosion with severe inflammatory cell infiltration and thickening of the synovial membrane; and Figure 1c shows an initially affected paw joint from a C5 blocker treated mouse showing preserved joint structure with some degree of synovial membrane thickening and a surprising lack of polymorphonuclear cell infiltration compared with the initially affected joint from the control group (Figure 1b).

Figures 2a-b are plots demonstrating the ability of a C5 blocker to stop the progression of pathology of an inflamed joint. Figure 2a shows a comparison of mean joint inflammation (JI) index values. Data represent

Samsung Bioepis Exhibit No. 1002
Page 8782

mean JI index +/- standard error. Solid circles are C5 blocker treated mice (n=6). Open circles are control treated mice (n=4). Figure 2b shows a comparison of paw thickness of initially affected joints. After onset of joint inflammation, control paw thickness increased significantly compared to treated or normal (N) paws. Data represent mean thickness +/- SE. Solid circles are C5 blocker treated mice (n=8). Open circles are control treated mice (n=4).

Figures 3a-e are plots of paw thickness versus time for C5 blocker treated and control treated mice. Figures 3a-d show measurements from initially affected paws of paired animals; Figure 3e shows measurements from initially affected paws of two unpaired treated animals and the mean control measurements of Figure 2b.

Figures 4a-c are photomicrographs of joints stained with Hematoxylin and Eosin illustrating the use of a C5 blocker to prevent the onset of joint inflammation. Figure 4a shows a paw joint from a normal mouse; Figure 4b shows an affected paw joint from a control treated mouse, where inflammatory cell infiltration, thickening of the synovial membrane and bone erosion by the expanding synovial pannus are visible; and Figure 4c shows a typical paw joint from a C5 blocker treated mouse showing only subclinical thickening of the synovial membrane.

Figure 5 shows that treatment with a C5 blocker prevents joint inflammation. Figure 5a is a comparison of the incidence of Collagen-Induced joint inflammation in C5 blocker treated mice (n=9) and in a pool of mice treated with two different control treatments (n=10). The data represent the overall (total) incidence of joint inflammation seen within two months after the initiation of treatment. Figure 5b is a comparison of serum hemolytic activity of C5 blocker treated mice (n=5) and control treated mice (n=3) two weeks after the initiation of treatment.

Samsung Bioepis Exhibit No. 1002
Page 8783

Figure 6 shows the effects of C5 blocker treatment on collagen-specific humoral and cellular responses. Figure 6a was prepared by analyzing serum samples obtained from C5 blocker treated (n=4) or control treated mice (n=4) at the indicated time after CII immunization. The samples were analyzed for anti B-CII IgG in an ELISA assay. The anti B-CII antibody index represents the ratio of optical densities obtained from positive serum and negative sera. Figure 6b was prepared by culturing lymph node T cells (5×10^5) in vitro with 20μg/ml B-CII, C-CII, B-CI or medium only, in the presence of 5×10^5 of mitomycin C treated syngeneic spleen cells for a total of 4 days, following which the cells were pulsed with ^{3}H-thymidine, 18 hours before harvest.

Figure 7 shows the results of hemolytic assays demonstrating inhibition of complement activity associated with human blood circulated through an extracorporeal circuit following treatment with a C5 blocker. Assays were performed before the addition of the blocker or the commencement of the CPB circuit ("Pre Tx") using undiluted blood ("undil") and diluted blood ("dil") as described in Example 4. Samples of diluted blood to which the blocker had been added ("Post Tx") were assayed at the times indicated after starting the CPB circuit.

Figure 8 shows the results of assays of levels of complement component C3a demonstrating that the generation of complement component C3a in whole human blood circulated through an extracorporeal circuit is not inhibited by the addition of a C5 blocker to such whole blood. Assays were performed before the addition of the blocker or the commencement of the CPB circuit ("Pre Tx") using undiluted blood ("undil") and diluted blood ("dil") as described in Example 5. Samples of diluted blood to which the blocker had been added ("Post Tx") were assayed at the times indicated after starting the CPB circuit.

Samsung Bioepis Exhibit No. 1002
Page 8784

Figure 9 shows the results of assays of the levels of soluble C5b-9 (sC5b-9) in human blood circulated through an extracorporeal circuit demonstrating that the addition of a C5 blocker to such whole blood inhibits the formation of the C5b-9 terminal complement assembly. Assays were performed before the addition of the blocker or the commencement of the CPB circuit ("Pre Tx") using undiluted blood ("undil") and diluted blood ("dil") as described in Example 6. Samples of diluted blood to which the blocker had been added ("Post Tx") were assayed at the times indicated after starting the CPB circuit.

Figures 10a-b show pharmacokinetic analyses of the reduction of the cell lysis ability of mouse blood (Fig. 10a) or extracorporeally circulating human blood (Fig. 10b) after treatment with various C5 blockers. C5 Blocker B is monoclonal antibody BB5.1; C5 Blocker F is an Fab' fragment of monoclonal antibody BB5.1; C5 Blocker N is monoclonal antibody N19-8.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

As discussed above, the present invention relates to a method for treating established joint inflammation by the administration of a C5 blocker or a combination of C5 blockers to a patient in need of such treatment. As used herein, "established joint inflammation" means that the patient has at least one inflamed joint at the time treatment is commenced.

Such C5 blockers comprise proteins (including antibodies), peptides, and other molecules that directly interact with C5, C5a, and/or C5b, so as to inhibit the formation of and/or physiologic function of C5a and/or C5b. Examples of non-protein molecules of this type include K-76 COOH, (see Hong et al., 1979), and substituted dihydrobenzofurans, spirobenzofuran-2(3H)-cycloalkanes, and their open chain intermediates, (see Sindelar et al., U.S. patent No. 5,173,499), that are reported to directly interact with C5, C5a, and/or C5b. Preferably, the C5 blocker or blockers inhibit the

Samsung Bioepis Exhibit No. 1002
Page 8785

formation of and/or physiologic function of both C5a and C5b.

The concentration and/or physiologic activity of C5a and C5b in a body fluid can be measured by methods well known in the art. For C5a such methods include chemotaxis assays, RIAs, or ELISAs (see, for example, Ward and Zvaifler, 1971; Jose et al., 1990; Wurzner et al., 1991). For C5b, hemolytic assays or assays for soluble C5b-9 as discussed herein can be used. Other assays known in the art can also be used. Using assays of these or other suitable types, candidate C5 blockers, now known or subsequently identified, can be screened in order to 1) identify compounds that are useful in the practice of the invention and 2) determine the appropriate dosage levels of such compounds. Examples 2, 4, and 6-8 illustrate the use of hemolytic and soluble C5b-9 assays with C5 blockers comprising monoclonal antibodies.

Blockers affecting C5a are preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25%) in the C5a levels present in at least one blood-derived fluid of the patient, e.g., blood, plasma, or serum, following activation of complement within the fluid. Alternatively, they are used at concentrations providing at least about a 10% reduction in the C5a levels present in the synovial fluid of an inflamed joint.

Similarly, blockers affecting C5b are preferably used at concentrations providing substantial reduction (i.e., reduction by at least about 25%) in the C5b levels present in at least one blood-derived fluid of the patient following activation of complement within the fluid. Alternatively, they are used at concentrations providing at least about a 10% reduction in the C5b levels present in the synovial fluid of an inflamed joint. In the case of C5b, such concentrations can be conveniently determined by measuring the cell-lysing

Samsung Bioepis Exhibit No. 1002
Page 8786

ability (e.g., hemolytic activity) of complement present in the fluid or the levels of soluble C5b-9 present in the fluid (see, for example, Example 6 below). Accordingly, a preferred concentration for a C5 blocker that affects C5b is one that results in a substantial reduction (i.e., a reduction by at least about 25%) in the cell-lysing ability of the complement present in at least one of the patient's blood-derived fluids. Reductions of the cell-lysing ability of complement present in the patient's body fluids can be measured by methods well known in the art such as, for example, by a conventional hemolytic assay such as the hemolysis assay described by Kabat and Mayer, 1961, pages 135-139, or a conventional variation of that assay such as the chicken erythrocyte hemolysis method described below.

Preferred C5 blockers are relatively specific, and do not block the functions of early complement components. In particular, such preferred agents will not substantially impair the opsonization functions associated with complement component C3b, which functions provide a means for clearance of foreign particles and substances from the body.

C3b is generated by the cleavage of C3, which is carried out by classical and/or alternative C3 convertases, and results in the generation of both C3a and C3b. Therefore, in order not to impair the opsonization functions associated with C3b, preferred C5 blockers do not substantially interfere with the cleavage of complement component C3 in a body fluid of the patient (e.g., serum) into C3a and C3b. Such interference with the cleavage of C3 can be detected by measuring body fluid levels of C3a and/or C3b, which are produced in equimolar ratios by the actions of the C3 convertases. Such measurements are informative because C3a and C3b levels will be reduced (compared to a matched sample without the C5 blocker) if cleavage is interfered with by a C5 blocker.

Samsung Bioepis Exhibit No. 1002
Page 8787

In practice, the quantitative measurement of such cleavage is generally more accurate when carried out by the measurement of body fluid C3a levels rather than of body fluid C3b levels, since C3a remains in the fluid phase whereas C3b is rapidly cleared. C3a levels in a body fluid can be measured by methods well known in the art such as, for example, by using a commercially available C3a EIA kit, e.g., that sold by Quidel Corporation, San Diego, CA, according to the manufacturers specifications. Particularly preferred C5 blockers produce essentially no reduction in body fluid C3a levels following complement activation when tested in such assays.

Preferred C5 blocking agents include antibodies. The antibodies are preferably monoclonal, although polyclonal antibodies, which can be produced and screened by conventional techniques, can also be used if desired. Hybridomas producing monoclonal antibodies (mAbs) reactive with complement component C5 can be obtained using complement component C5, C5a, and/or C5b, preferably in purified form, as the immunogen.

The most preferred antibodies will prevent the cleavage of C5 to form C5a and C5b, thus preventing the generation of the anaphylatoxic activity associated with C5a and preventing the assembly of the membrane attack complex associated with C5b. As discussed above, in a particularly preferred embodiment, these C5 blocking antibodies will not impair the opsonization function associated with the action of C3b.

General methods for the immunization of animals (in this case with C5, C5a, and/or C5b), isolation of antibody producing cells, fusion of such cells with immortal cells (e.g., myeloma cells) to generate hybridomas secreting monoclonal antibodies, screening of hybridoma supernatants for reactivity of secreted monoclonal antibodies with a desired antigen (in this case the immunogen or a molecule containing the

Samsung Bioepis Exhibit No. 1002
Page 8788

immunogen), the preparation of quantities of such antibodies in hybridoma supernatants or ascites fluids, and for the purification and storage of such monoclonal antibodies, can be found in numerous publications. These include: Coligan, et al., eds. Current Protocols In Immunology, John Wiley & Sons, New York, 1992; Harlow and Lane, Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Liddell and Cryer, A Practical Guide To Monoclonal Antibodies, John Wiley & Sons, Chichester, West Sussex, England, 1991; Montz, et al., Cellular Immunol. 127:337-351, 1990; Wurzner, et al., Complement Inflamm. 8:328-340, 1991; and Mollnes, et al., Scand. J. Immunol. 28:307-312, 1988.

A description of the preparation of a mouse anti-human-C5 monoclonal antibody with preferred binding characteristics is presented below in Example 8. Wurzner, et al., 1991, describe the preparation of other suitable mouse anti-human-C5 monoclonal antibodies referred to as N19-8 and N20-9.

As used herein, the terms "antibody" or "antibodies" refer to immunoglobulins produced in vivo, as well as those produced in vitro by a hybridoma, and antigen binding fragments (e.g., Fab' preparations) of such immunoglobulins, as well as to recombinantly expressed (engineered) antigen binding proteins, including immunoglobulins, chimeric immunoglobulins, "humanized" immunoglobulins, antigen binding fragments of such immunoglobulins, single chain antibodies, and other recombinant proteins containing antigen binding domains derived from immunoglobulins. As used herein, the term "monoclonal" refers to any antibody that is not of polyclonal origin.

Publications describing methods for the preparation of engineered antibodies, in addition to those listed immediately above, include: Reichmann, et al., Nature, 332:323-327, 1988; Winter and Milstein, Nature, 349:293-299, 1991; Clackson, et al., Nature, 352:624-628, 1991;

Samsung Bioepis Exhibit No. 1002
Page 8789

Morrison, Annu Rev Immunol, 10:239-265, 1992; Haber, Immunol Rev, 130:189-212, 1992; and Rodrigues, et al., J Immunol, 151:6954-6961, 1993. Human or humanized antibodies are preferred for administration to human patients.

To achieve the desired reductions of body fluid parameters, such anti-C5 antibodies can be administered in a variety of unit dosage forms. The dose will vary according to the particular antibody. For example, different antibodies may have different masses and/or affinities, and thus require different dosage levels. Antibodies prepared as Fab' fragments or single chain antibodies will also require differing dosages than the equivalent native immunoglobulins, as they are of considerably smaller mass than native immunoglobulins, and thus require lower dosages to reach the same molar levels in the patient's blood.

Dosage levels of the antibodies for human subjects are generally between about 0.5 mg per kg and about 100 mg per kg per patient per treatment, and preferably between about 1 mg per kg and about 50 mg per kg per patient per treatment. In terms of body fluid concentrations, the antibody concentrations are preferably in the range from about 5 μg/ml to about 500 μg/ml.

Other C5 blockers can also be administered in a variety of unit dosage forms and their dosages will also vary with the size, potency, and in vivo half-life of the particular C5 blocker being administered.

Doses of C5 blockers will also vary depending on the manner of administration, the particular symptoms of the patient being treated, the overall health, condition, size, and age of the patient, and the judgment of the prescribing physician.

Subject to the judgement of the physician, a typical therapeutic treatment includes a series of doses, which will usually be administered concurrently with the

Samsung Bioepis Exhibit No. 1002
Page 8790

monitoring of clinical endpoints such as number of joints involved, redness of joints, swelling of joints, mobility of joints, pain levels, etc., with the dosage levels adjusted as needed to achieve the desired clinical outcome.

The frequency of administration may also be adjusted according to various parameters. These include the clinical response, the plasma half-life of the C5 blocker, and the levels of the blocker in a body fluid, such as, blood, plasma, serum, or synovial fluid. To guide adjustment of the frequency of administration, levels of the C5 blocker in the body fluid may be monitored during the course of treatment.

Alternatively, for C5 blockers that affect C5b, levels of the cell-lysing ability of complement present in one or more of the patient's body fluids are monitored to determine if additional doses or higher or lower dosage levels are needed. Such doses are administered as required to maintain at least about a 25% reduction, and preferably about an 50% or greater reduction of the cell-lysing ability of complement present in blood, plasma, or serum, or at least about a 10% reduction of the cell-lysing ability of complement present in synovial fluid from an inflamed joint. The cell-lysing ability can be measured as percent hemolysis in hemolytic assays of the types described herein. A 10% or 25% or 50% reduction in the cell-lysing ability of complement present in a body fluid after treatment with the C5 blocker or blockers used in the practice of the invention means that the percent hemolysis after treatment is 90, 75, or 50 percent, respectively, of the percent hemolysis before treatment. In yet another alternative, dosage parameters are adjusted as needed to achieve a substantial reduction of C5a levels in blood, plasma, or serum, or at least a 10% reduction of the C5a levels in the synovial fluid of an inflamed joint. As discussed above, C5a levels can be measured using the techniques

Samsung Bioepis Exhibit No. 1002
Page 8791

described in Wurzner, et al., Complement Inflamm 8:328-340, 1991. Other protocols of administration can, of course, be used if desired as determined by the physician.

In a preferred embodiment, administration of the C5 blocker is initiated when the patient experiences a "flare up" of joint inflammation in which one or more affected joints becomes more swollen and takes on an erythematous (reddened) appearance.

Administration of the C5 blockers will generally be performed by a parenteral route, typically via injection such as intra-articular or intravascular injection (e.g., intravenous infusion) or intramuscular injection. Other routes of administration, e.g., oral (p.o.), may be used if desired and practicable for the particular C5 blocker to be administered.

For the treatment of established joint inflammation by systemic administration of a C5 blocker (as opposed to local administration, e.g., intra-articular injection into the inflamed joint) administration of a large initial dose is preferred, i.e., a single initial dose sufficient to yield a substantial reduction, and more preferably an at least about 50% reduction, in the hemolytic activity of the patient's serum. Such a large initial dose is preferably followed by regularly repeated administration of tapered doses as needed to maintain substantial reductions of serum hemolytic titer. In another embodiment, the initial dose is given by both local and systemic routes, followed by repeated systemic administration of tapered doses as described above.

Formulations suitable for injection, p.o., and other routes of administration are well known in the art and may be found, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Company, Philadelphia, PA, 17th ed. (1985). Parenteral formulations must be sterile and non-pyrogenic, and generally will include a pharmaceutically effective carrier, such as saline,

Samsung Bioepis Exhibit No. 1002
Page 8792

buffered (e.g., phosphate buffered) saline, Hank's solution, Ringer's solution, dextrose/saline, glucose solutions, and the like. These formulations may contain pharmaceutically acceptable auxiliary substances as required, such as, tonicity adjusting agents, wetting agents, bactericidal agents, preservatives, stabilizers, and the like.

The formulations of the invention can be distributed as articles of manufacture comprising packaging material and a pharmaceutical agent which comprises the C5 blocker or blockers and a pharmaceutically acceptable carrier as appropriate to the mode of administration. The packaging material will include a label which indicates that the formulation is for use in the treatment of joint inflammation and may specifically refer to arthritis, rheumatoid arthritis, osteoarthritis, lupus arthritis, psoriatic arthritis, juvenile onset rheumatoid arthritis, reactive arthritis, Reiter's syndrome (Reiter's disease), or other diseases involving joint inflammation.

Without intending to limit it in any manner, the present invention will be more fully described by the following examples. The methods and materials which are common to various of the examples are as follows.

MATERIALS AND METHODS

Cell Lysis Assays

The cell-lysing ability of complement in various body fluid samples was determined using hemolytic assays performed as follows: Chicken erythrocytes were washed well in GVBS (Sigma Chemical Co. St. Louis, MO, catalog No. G-6514) and resuspended to $2x10^8$/ml in GVBS. Anti-chicken erythrocyte antibody (IgG fraction of anti-chicken-RBC antiserum, Intercell Technologies, Hopewell, NJ) was added to the cells at a final concentration of 25 μg/ml and the cells were incubated for 15 min. at 23°C. The cells were washed 2x with GVBS and $5x10^6$ cells were resuspended to 30 μL in GVBS. A 100 μL volume of body fluid test solution was then added to yield a final

Samsung Bioepis Exhibit No. 1002
Page 8793

reaction mixture volume of 130 μL. As used herein, reference to the serum percentage and/or serum input in these assays indicates the percent of a body fluid (including serum, as well as other body fluids such as blood, plasma, or synovial fluid) in the 100 μL volume of body fluid test solution.

After incubation for 30 min. at 37°C, percent hemolysis was calculated relative to a fully lysed control sample. Hemolysis was determined by spinning the cells down and measuring released hemoglobin in the supernatant as the optical density at 415nm.

A 50% reduction in hemolysis after treatment with the C5 blocker or blockers used in the practice of the invention means that the percent hemolysis after treatment was one half of the percent hemolysis before treatment.

Various hemolytic assays described below in the examples were performed using this chicken erythrocyte assay with the following body fluid inputs. For assays of mouse complement activity, the 100 μL volume of body fluid test solution contained 50 μL of diluted (in GVBS) mouse serum and 50 μL of human C5 deficient serum (Quidel Corporation, San Diego, CA). For assays of human complement activity, the body fluid test solution contained various concentrations of human plasma or serum, with hybridoma supernatants and/or GVBS being added to yield the final 100 μL volume. For the assays used to screen hybridoma supernatants discussed below in example 8 each 100μL volume of serum test solution contained 50μL of hybridoma supernatant and 50μL of a 10% solution of human serum in GVBS, yielding a 5% human serum input.

Collagen Induced Joint Inflammation

Examples 1, 2, and 3 use a collagen induced joint inflammation system that has been employed since the 1970s as an animal model of human joint inflammation, particularly RA (see, for example, Trentham et al., 1977;

Samsung Bioepis Exhibit No. 1002
Page 8794

Holmdahl et al., 1986; Boissier et al., 1987; Yoo et al., 1988). This model system was implemented using 8-12 week old male DBA/1LacJ mice that were purchased from The Jackson Laboratory (Bar Harbor, ME).

Immunization

Bovine collagen II (B-CII) obtained from Elastin Products Company, Inc., Owensville, MO, was dissolved in 0.01M acetic acid by stirring overnight at 4°C at a concentration of 4mg/ml. Complete Freund's adjuvant (CFA) was prepared by the addition of desiccated Mycobacterium tuberculosis H37RA (Difco, Detroit, MI) to incomplete Freunds adjuvant (Difco) at a concentration of 2mg/ml. The solution of B-CII was emulsified in an equal volume of CFA and a 100μl aliquot of this emulsion, containing 200μg B-CII and 100μg of Mycobacterium, was injected intradermally at the base of the mouse's tail. After 21 days, all mice were reimmunized using the identical protocol. This secondary CII reimmunization served primarily to boost the serum levels of anti-CII antibodies in the immunized mice. After CII reimmunization, the onset of joint inflammation (JI) and disease progression rise dramatically, characterized by the severe swelling and redness of the joints of one or more paws at around 4-6 weeks after the initial immunization.

Clinical evaluation

Mice were examined daily beginning on the day of reimmunization for the appearance of JI. The presence of JI was determined by examining, measuring and scoring each of the forepaws and hindpaws. Collagen induced JI (CIJI) is characterized by swelling and erythema or visible joint distortion of one or more extremities. The severity of JI in each affected paw was scored as: 0 - normal joint; 1 - visible redness and swelling; 2 - severe redness and swelling affecting entire paw or joint; or 3 - deformed paw or joint with ankylosis. The sum of the scores for all four paws in each mouse was

Samsung Bioepis Exhibit No. 1002
Page 8795

used as an index (the "JI index") to assess overall disease severity and progression.

Anti-complement Monoclonal Antibodies

Monoclonal antibodies that bind to and block mouse C5 were prepared by standard methods from hybridoma BB5.1 (Frei, et al., 1987), which was obtained from Dr. Brigitta Stockinger of the National Institute for Medical Research, Mill Hill, London, England. Anti-human C8 hybridoma, 135.8, which generates an Mab that does not block mouse C8, was obtained from Dr. Peter Sims (Blood Research Institute, Milwaukee, WI). Both antibodies are IgG1 isotypes, and ascites of BB5.1 or 135.8 were obtained in athymic nude mice or BALB/c mice, respectively. IgGs were purified from ascites with a protein A affinity collum eluted with acetic acid, and subsequently dialyzed against PBS. Purified antibodies were quantified by spectrophotometric determination of absorbance at 280nm and sterilized with a 0.22 μm filter.

Antibody administration

For prophylactic treatment, mice were randomly divided as C5 blocker treated and control treated groups and subsequently received 750μg per mouse ip doses of either anti-mouse C5 mAb, BB5.1, or anti-human C8 mAb, 135.8, as a control, twice weekly. For therapeutic treatment of established JI, mice received anti-mouse C5 mAb BB5.1 or anti-human C8 mAb 135.8 at 2-5 mg/mouse ip daily for 10 days after the initial onset of JI was observed. The doses of anti-C5 mAb were adjusted in a range spanning 2 mg to 5 mg per injection to ensure that the desired depletion of C5 mediated hemolytic activity was obtained, i.e., a depletion of at least 50%.

Unlike the situation in humans, where administration of a C5 blocker to a body fluid of genetically unrelated individuals results in roughly equivalent levels of complement inhibition, the dose of anti-murine-C5 mAb required to deplete hemolytic activity by a given amount in mice is strain dependent. The dose required to

Samsung Bioepis Exhibit No. 1002
Page 8796

deplete hemolytic activity in DBA/1LacJ mice is approximately four times higher than the dose required to achieve an equivalent depletion of the hemolytic activity in BALB/c mice.

T cell stimulation assays

Lymph node cells taken from animals at the time of sacrifice were analyzed for specific T cell responses to collagen II. T cells (5×10^5) were incubated with 5×10^5 mitomycin C (50μg/ml) treated syngeneic spleen cells from normal DBA/1LacJ mice in flat bottomed 96-well plates. Bovine collagen II (B-CII), bovine CI (B-CI), chicken CII (C-CII) and ovalbumin or BSA were added to cultures at 20μg/ml. The culture medium was RPMI-1640 supplemented with 5% heated inactivated FCS, 5×10^{-5} M 2-ME, 10mM HEPES buffer, 1% L-glutamine, 1% sodium pyruvate, and 1% penstrep. The cultures were incubated at 37°C in 5% CO_2 for 4 days. Eighteen hours before harvesting, 1μci of ^{3}H-thymidine was added to each well. Results are expressed as cpm obtained from triplicate T cell cultures.

Quantification of anti B-CII antibodies

Mice were bled at various times after immunization with B-CII. Serum anti B-CII antibody titers were measured using a conventional ELISA for B-CII similar to the ELISA for anti C5 antibodies described below in Example 8, but using B-CII to coat the plates (also see Myers, et al., 1989, and Seki et al., 1992, for descriptions of similar assays).

Histological examination

Mice from each group were sacrificed and all four legs from each mouse were fixed in 10% buffered formalin and decalcified in a solution of 3.1% HCL, 5% formic acid and 7% aluminum chloride. The tissue samples were embedded in paraffin, sectioned at 5μm and stained with hematoxylin and eosin. For immunofluorescence staining, paws were decalcified in a 0.1M Tris solution containing 10% EDTA and 7.5% PVP for 3 days and frozen in OCT at

Samsung Bioepis Exhibit No. 1002
Page 8797

-80°C. 5μm sections were then prepared and stained with 9FITC conjugated goat anti-mouse IgG, IgA, and IgM (Zymed Laboratories, South San Francisco, CA, Catalog No. 65-6411) at a dilution of 1 to 50.

EXAMPLE 1

Therapeutic Effects of C5 Blocker Treatment after the Clinical Onset of Collagen Induced Joint Inflammation

In order to assess the effects of administration of a C5 blocker on established JI, mice were observed following induction of CIJI, as described above, and pairs of mice were selected that showed the initial appearance of readily detectable JI symptoms (swollen joints of the paws) on the same day. One mouse of each such pair was treated with a anti-C5 mAb BB5.1 and one was treated with a control injection of either the irrelevant mAb 135.8 or PBS. In each matched pair, the animal with the greatest overall level of paw inflammation was assigned to the C5 blocker treatment group so as to avoid potentially biasing the results in favor of the C5 blocker treatment. Starting on the first day when joint inflammation was observed as paw inflammation, treatments were continued daily for 10 days. (In one case a pair of mice was only carried for 8 days.) In addition to these matched pairs, two unpaired animals also received the C5 blocker treatment after the induction of JI.

Histological examination of initially affected joints from control-treated mice at the end of the treatment period revealed extensive bone erosion with severe inflammatory cell infiltration, thickening of the synovial membranes, and pannus formation (Figure 1b). In contrast, the initially affected joints of the C5 blocker treated group showed preserved joint structure with some degree of thickening of synovial membranes and mononuclear cell infiltration into some of the joints (Figure 1c). The severe inflammatory cell infiltration in the control-treated joints was predominantly made up

Samsung Bioepis Exhibit No. 1002
Page 8798

of polymorphonuclear cells (PMNs, neutrophils). Surprisingly, such PMN infiltration was almost completely absent in the C5 blocker treated mice.

During the clinical course of CIJI, an important indicator of the progression of disease is the involvement of additional limbs. Therefore, the number of limbs with clinically detectable JI at the end of the treatment period was compared with the number of limbs exhibiting JI symptoms before the start of therapy. The severity and progression of JI in each affected paw was determined and scored as described above under the heading "Materials and Methods", and the sum of the scores for all four paws of each animal was used as a "JI index". The thicknesses of all four paws of each animal were also measured with a caliper during the time of this experiment to provide a completely objective evaluation of this aspect of disease progression.

As shown in Table 1 (mean values) and Table 2 (individual values), there were significant increases in new limb involvement in the control treated group during the course of 10 day treatment, while the number of inflamed limbs was decreased when DBA/1LacJ mice with inflamed joints were treated with the C5 blocker starting at the time of disease onset. In addition to new limb recruitment, the initially affected paws of the control treated animals evidenced progression of inflammatory joint disease severity by becoming more inflamed (Figure 2a). Acute inflammation in the affected joints was observed as severe joint swelling and redness during the first few days, followed by joint deformation and ankylosis at the end of 10 day period. In contrast, no new paws were involved and the severity of inflammation in the majority of affected joints subsided or remained unchanged during the course of C5 blocker therapy (Figure 2a).

The paw thicknesses of initially affected limbs in both C5 blocker treated and control treated groups during

Samsung Bioepis Exhibit No. 1002
Page 8799

the course of these experiments is shown as mean values for each group in Figure 2b. Figures 3a, 3b, 3c, and 3d show values for each initially inflamed paw of each of the matched pairs of control treated and C5 blocker treated animals, while Figure 3e shows the values obtained for each initially inflamed paw of each of the unpaired C5 blocker treated animals (shown along with the mean values for control treated animals of Figure 2b). In these figures, the number in parenthesis indicates the designation of the particular animal, while the letters following the numbers (only in those cases where more than one limb was affected initially) indicate the particular paws affected, with the first letter indicating front (F) or rear (R) paws, and the second letter indicating right (R) or left (L) paws.

As can be seen in Figures 2 and 3, and in Tables 1 and 2, C5 blocker treatment successfully prevented further paw recruitment and reduced (but did not completely abolish) the inflammation in the initially affected joints in all but one (mouse #4) of the C5 blocker treated animals. As can be seen in Figure 2b, the mean thickness of initially affected paws in the control treated group increased significantly during the 10 day period, while the mean thickness of initially affected paws in the C5 blocker treated group decreased, but not significantly.

EXAMPLE 2

Prophylactic Treatment with a C5 Blocker Prevents Collagen Induced Joint Inflammation

In these experiments, the administration of the C5 blocker coincided with the reimmunization of the experimental animals with B-CII. On the day of reimmunization, mice were symptom free, and were randomly assigned to C5 blocker treatment or control treatment groups. Each mouse was treated with either the C5 blocker (anti-mouse C5 mAb, BB5.1) or a control treatment (anti-human C8 mAb, 135.8) at 750μg per mouse ip twice

Samsung Bioepis Exhibit No. 1002
Page 8800

weekly. The animals were treated for four weeks, at which time treatment was discontinued. The results of this study are shown in Figures 4 and 5.

Administration the C5 blocker completely prevented the development of CIJI (0/8). All mice in the C5 blocker treated group exhibited no signs of clinical disease during the period of treatment (and for up to two months after discontinuing the C5 blocker therapy in the two animals followed for that long). In contrast, 90% of the control treated animals (9/10) developed JI by 4-6 weeks after the first B-CII immunization. The percent incidence of JI observed in the control treated and C5 blocker treated animals after 4-6 weeks is plotted in Figure 5a. (Note that the value for the C5 blocker treated group in this figure is actually 0%, but a bar indicating 1% has been plotted in order to indicate that the data for this set of animals was obtained and is presented.) Peak inflammation levels were observed around 5 weeks after the initial collagen immunization. As shown in Figure 5b, 80% to 90% of the serum hemolytic activity was depleted in the C5 blocker treated group, while the serum hemolytic activity remained normal in the control treated group.

As shown in Figure 4, histological examination of affected joints from control mice revealed extensive mononuclear cell as well as polymorphonuclear cell infiltration, thickening of the synovial membrane and bone erosion by the expanding synovial pannus (Figure 4b). In contrast, there were no signs of inflammatory processes observed in the majority of joints studied from the C5 blocker treated mice. A few joints from these C5 blocker treated mice showed some subclinical thickening of the synovial membrane, but this alteration was not accompanied by any visible bone erosion or inflammatory cell infiltration (Figure 4c). Interestingly, immunofluorescence staining showed antibody deposition along cartilage surfaces and C3 activation at synovial

Samsung Bioepis Exhibit No. 1002
Page 8801

membranes in the joints of both the control treated and the C5 blocker treated animals.

EXAMPLE 3

Effect of C5 Blocker Treatment on the Humoral and Cellular Immune Responses to Immunization with Collagen

Responses of both the humoral and cellular immune systems are activated after immunization of DBA/1LacJ mice with bovine Collagen II. Anti B-CII titers increase, and the serum IgG anti B-CII titers in C5 blocker treated mice are equivalent to those of control treated mice when tested at 14, 28 and 42 days after the initial B-CII immunization (Figure 6a). Anti B-CII antibody titers from both control and anti-C5 mAb treated mice rise significantly after the B-CII reimmunization and remain at the resulting plateau for an extended period of time.

In order to study T cell responses, lymph node cells (LNCs) from C5 blocker treated mice and control treated mice were cultured with either B-CII, B-CI, C-CII, or culture medium only. LNCs from either C5 blocker treated mice or control treated mice responded specifically and equally to B-CII regardless of the treatment the animals received concurrently with B-CII reimmunization. C-CII, which shares many conserved regions of homology with B-CII also elicited a moderate T cell response when cultured with LNCs from C5 blocker treated mice or from control treated mice. In contrast, LNCs from age matched non-immunized mice responded poorly to all of the tested collagens (Figure 6b).

The data obtained in these experiments and those of Examples 1 and 2 clearly demonstrate that in vivo administration of a C5 blocker prevents the development and progression of CIJI and that this treatment does not interfere with the humoral and cellular immune responses seen after immunizing mice with bovine type II collagen. Both collagen-specific T cell responses and anti-CII antibody titers were comparable in both the C5 blocker

Samsung Bioepis Exhibit No. 1002
Page 8802

treated mice and the control treated B-CII reimmunized mice.

EXAMPLE 4

C5 Blocker Inhibition of Complement Activity

The effects of a C5 blocker on complement activation were evaluated using a closed-loop cardio-pulmonary bypass (CPB) model for the extracorporeal circulation of human blood. As discussed fully in copending U.S. patent application Serial No. 08/217,391, filed March 23, 1994, extracorporeal circulation of human blood causes activation of complement in the blood.

The C5 blocker was a monoclonal antibody raised in mice against purified human C5 protein (Wurzner, et al., Complement Inflamm 8:328-340, 1991; mAb N19-8) that was propagated, recovered and purified as an IgG fraction from mouse ascites fluid (Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory, New York, 1988; Current Protocols In Immunology, John Wiley & Sons, New York, 1992).

To carry out these experiments, 300 ml of whole human blood was drawn from a healthy human donor and additionally a 1 ml sample was removed as a control sample for later analysis. The blood was diluted to 600 ml by the addition of Ringer's lactate solution containing 10U/ml heparin. The C5 blocker (30 mg in sterile PBS) was added to the diluted blood to a final concentration of 50 μg/ml. In a control experiment, an equal volume of sterile PBS was added to the diluted blood. The blood was then used to prime the extracorporeal circuit of a COBE CML EXCEL membrane oxygenator CPB machine (Cobe BCT, Inc., Lakewood, CO) and the circuit was started. The circuit was cooled to 28°C and circulated for 60 minutes. The circuit was then warmed to 37°C and circulated for an additional 30 minutes. The experiment was then terminated. Samples were taken at several time points and evaluated for complement activity (Figure 7).

Samsung Bioepis Exhibit No. 1002
Page 8803

At each time point an aliquot of white blood was taken, divided into 3 samples and A) diluted 1:1 in 2% paraformaldehyde in PBS to evaluate platelet and blood cell activation parameters as discussed in the above-referenced U.S. Patent Application Serial No. 08/217,391; B) centrifuged to remove all cells and plasma diluted 1:1 in Quidel sample preservation solution (Quidel Corporation, San Diego, CA) and stored at -80°C, following which these frozen diluted plasma samples were thawed and used to evaluate C3a and C5b-9 generation (Examples 5 and 6, respectively), and C) centrifuged to remove all cells and undiluted plasma stored at -80°C, following which these frozen plasma samples were thawed and hemolytic assays were performed as described above.

As can be seen in Figure 7, addition of the C5 blocker to the extracorporeal circuit resulted in a 95% reduction of the cell-lysing ability of complement in the plasma. The complement activity remained inhibited throughout the course (90 minutes) of the experiment.

EXAMPLE 5

Generation of C3a in the Presence of a C5 Blocker

The fresh frozen plasma samples that had previously been diluted in Quidel sample preservation solution following CPB circulation (see Example 4) were assayed for the presence of the complement split product C3a by using the Quidel C3a EIA kit (Quidel Corporation, San Diego, CA). These measurements were carried out according to the manufacturer's specifications. C3a released is expressed in ng/well as determined by comparison to a standard curve generated from samples containing known amounts of human C3a.

As seen in Figure 8, addition of the C5 blocker had no effect on the production of C3a during the CPB experiment. C3a generation was dramatically increased during the final 30 min. of the experiment and correlates with sample warming.

Samsung Bioepis Exhibit No. 1002
Page 8804

EXAMPLE 6

Prevention of the Generation of C5b-9 by a C5 Blocker

Fresh frozen plasma samples that had been previously diluted in Quidel sample preservation solution following CPB circulation (see Example 4) were assayed for the presence of the terminal human complement complex C5b-9 using the Quidel C5b-9 kit (Quidel Corporation, San Diego, CA). The amount of soluble C5b-9 (sC5b-9) in each sample was determined using the manufacturers specifications and is expressed in arbitrary absorbance units (AU).

As can be seen in Figure 9, the C5 blocker completely inhibited C5b-9 generation during extracorporeal circulation so that sC5b-9 levels during the full course of the run were equivalent to control (t_0) time points. The results of this experiment and those of Examples 4 and 5 show that the addition of a C5 blocker to human blood undergoing extracorporeal circulation effectively inhibits both complement hemolytic activity (Figure 7) and C5b-9 generation (Figure 9), but not C3a generation (Figure 8).

Example 7

Pharmacokinetics of mAb C5 Blockers

The in vivo duration of action of mAb BB5.1, and a Fab' fragment of mAb BB5.1 (prepared by standard methods) was determined in normal female BALB/cByJ mice (averaging approximately 20 gms each) which were obtained from the Jackson Laboratory, Bar Harbor, ME. The mice were given a single intravenous injection (at 35 mg/kg body weight) of the mAb or the Fab' fragment of the mAb (or an equal volume of PBS as a control). Blood samples were collected from the retroorbital plexus at 1, 4, 24, 96, and 144 hours after administration of PBS; 4, 16, and 24 hours after administration of the Fab' fragment of mAb BB5.1; and 4, 24, 48, 72, 96, and 144 hours after administration of intact mAb BB5.1.

Samsung Bioepis Exhibit No. 1002
Page 8805

Fig. 10a shows the time course of inhibition of the cell-lysing ability of complement in mouse blood (determined by testing serum obtained from the blood and diluted to 2.5% in hemolytic assays, as described above) after the in vivo administration of the intact mAb, the Fab' fragment, or the PBS. As shown in the figure, the intact mAb almost completely inhibited the hemolytic activity of the blood throughout the 6 day test period. The Fab', however, had a half-life of approximately 24 hours.

In addition to the above experiments, at the end of the 6 day testing period all of the mice were sacrificed. Kidneys, lungs, and livers were harvested and examined by gross inspection, as well as by microscopic examination of stained sections. All of the organs of the C5 blocker treated animals appeared the same as those taken from the PBS control treated animals. The overall appearance of these test and control mice was also indistinguishable prior to necropsy.

An anti-human C5 mAb was also tested for pharmacokinetic properties in circulating human blood as described above in Example 4. As described therein, the hemolysis-inhibiting effects of this C5 blocker were assayed over a 90 minute period of circulation. The results of these assays are charted in Figure 10b, and show that the C5 blocker essentially completely inhibited the cell lysing ability of the human blood during the entire 90 minute period of circulation.

The results of these experiments demonstrate that these C5 blockers will survive in the bloodstream for a substantial period of time, thus making periodic administration practical.

EXAMPLE 8

Preparation of a C5 Blocker

A C5 blocker mAb suitable for use in the practice of the present invention was prepared as follows.

Samsung Bioepis Exhibit No. 1002
Page 8806

Balb/c mice were immunized three times by intraperitoneal injection with human C5 protein (Quidel Corporation, San Diego, CA, Catalog #A403). The first injection contained 100µg of C5 protein in a complete Freund's adjuvant emulsion, the second immunization contained 100µg of C5 protein in an incomplete Freund's adjuvant emulsion, and the third immunization was 100µg of protein in PBS. The mice were injected at roughly 2 month intervals.

Fusions of splenocytes to myeloma cells to generate hybridomas were performed essentially as described in Current Protocols in Immunology (John Wiley & Sons, New York, 1992, pages 2.5.1 to 2.5.17). One day prior to fusion the mice were boosted IV with 100µg of C5 protein. On the day of fusion, the immunized mice were sacrificed and spleens was harvested. SP2/0-AG14 myeloma cells (ATCC CRL#1581) were used as the fusion partner. SP2/0-AG14 cultures were split on the day before the fusion to induce active cell division. A ratio of 1:10 (myeloma cells:splenocytes) was used in the fusions.

The cells were fused using PEG 1450 in PBS without calcium (Sigma Chemical Company, St. Louis, MO, Catalog No. P-7181) and plated at 1-2.5 x 10^5 cells per well. Selection in EX-CELL™ 300 medium (JRH Biosciences, Lexena, KS, Catalog No. 14337-78P) supplemented with 10% heat inactivated fetal bovine serum (FBS); glutamine, penicillin and streptomycin (GPS); and HAT (Sigma Chemical Company, St. Louis, MO, Catalog No. H-0262) was started the following day. The fusions were then fed every other day with fresh FBS, GPS, and HAT supplemented medium. Cell death could be seen as early as 2 days and viable cell clusters could be seen as early as 5 days after initiating selection. After two weeks of selection in HAT, surviving hybridomas chosen for further study were transferred to EX-CELL 300 medium supplemented with FBS, GPS, and HAT (Sigma Chemical Company, St. Louis, MO, Catalog No. H-0137) for 1 week and then cultured in

Samsung Bioepis Exhibit No. 1002
Page 8807

EX-CELL 300 medium supplemented with FBS and GPS.

Hybridomas were screened for reactivity to C5 and inhibition of complement-mediated hemolysis 10-14 days after fusion, and were carried at least until the screening results were analyzed. The screen for inhibition of hemolysis was the chicken erythrocyte lysis assay described above. The screen for C5 reactivity was an ELISA, which was carried out using the following protocol.

A 50 µL aliquot of a 2µg/ml solution of C5 (Quidel Corporation, San Diego, CA) in sodium carbonate/bicarbonate buffer, pH 9.5, was incubated overnight at 4°C in each test well of a 96 well plate (Nunc-Immuno™ F96 Polysorp™, A/S Nunc, Roskilde, Denmark). The wells were then subjected to a wash step. (Each wash step consisted of three washes with TBST.) Next, test wells were blocked with 200 µL of blocking solution, 1% BSA in TBS (BSA/TBS), for 1 hour at 37°C. After an additional wash step, a 50µL aliquot of hybridoma supernatant was incubated in each test well for 1 hour at 37°C with a subsequent wash step. As a secondary (detection) antibody, 50µL of a 1:2000 dilution of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG in BSA/TBS, was incubated in each test well for 1 hour at 37°C, followed by a wash step. Following the manufacturer's procedures, 10 mg of O-phenylenediamine (Sigma Chemical Company, St. Louis, MO, Catalog No. P-8287) was dissolved in 25 mLs of phosphate-citrate buffer (Sigma Chemical Company, St. Louis, MO, Catalog No. P-4922), and 50 µL of this substrate solution was added to each well to allow detection of peroxidase activity. Finally, to stop the peroxidase detection reaction, a 50µL aliquot of 3N hydrochloric acid was added to each well. The presence of antibodies reactive with C5 in the hybridoma supernatants was read out by a spectrophotometric OD determination at 490 nm.

Samsung Bioepis Exhibit No. 1002
Page 8808

The supernatant from a hybridoma designated as 5G1.1 tested positive by ELISA and substantially reduced the cell-lysing ability of complement present in normal human blood in the chicken erythrocyte hemolysis assay. Further analyses revealed that the 5G1.1 antibody reduces the cell-lysing ability of complement present in normal human blood so efficiently that, even when present at roughly one-half the molar concentration of human C5 in the hemolytic assay, it can almost completely neutralize serum hemolytic activity.

Hybridoma 5G1.1 was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, 20852, United States of America, on April 27, 1994, and has been assigned the designation HB-11625. This deposit was made under the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (1977).

Although preferred and other embodiments of the invention have been described herein, further embodiments may be perceived by those skilled in the art without departing from the scope of the invention as defined by the following claims.

Samsung Bioepis Exhibit No. 1002
Page 8809

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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
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Samsung Bioepis Exhibit No. 1002
Page 8812

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Samsung Bioepis Exhibit No. 1002
Page 8814

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TABLE 1

		NUMBER OF LIMBS AFFECTED PER GROUP*			
Treatment	n	Day 0	Day 10	% Change	% Hemolytic Activity
Control	4	4 (1.0)	9 (2.3)	+125.0	95.6 ± 3.8
C5 Blocker	6	8 (1.3)	7 (1.2)	- 12.5	13.9 ± 4.7

* Numbers in parenthesis represent the average number of affected joints per mouse.

Samsung Bioepis Exhibit No. 1002
Page 8816

TABLE 2

	NUMBER OF LIMBS AFFECTED PER MOUSE			
Mouse (treatment)	Day 0	Day 10	% Change	% Hemolytic Activity
#8 (control)	1 (RL)	2 (RL & RR)	+100	91.2
#2 (C5 blocker)	1 (FL)	1 (FL)*	0	22.4
#6 (control)	1 (RR)	2 (RR & RL)	+100	103.2
#5 (C5 blocker)	1 (RR)	1 (RR)	0	6.0
#1 (control)	1 (FL)	2 (FL & FR)	+100	92.4
#4 (C5 blocker)	1 (RL)	2 (FL & RL)	+100	9.2
#9 (control)†	1 (RL)	2 (RR & RL)	+100	not tested
#3 (C5 blocker)†	2 (FL & RR)	1 (FL)	-100	0.4
#7 (C5 blocker)§	1 (FR)	1 (FR)	0	13.6
#10 (C5 blocker)§	2 (RL & RR)	1 (RR)**	-100	32.0

* barely detectable inflammation

** only 1 toe

† data represent only 8 days for mouse #9 and #3

§ mouse #7 and #10 were not provided with matched controls

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Samsung Bioepis Exhibit No. 1002
Page 8817

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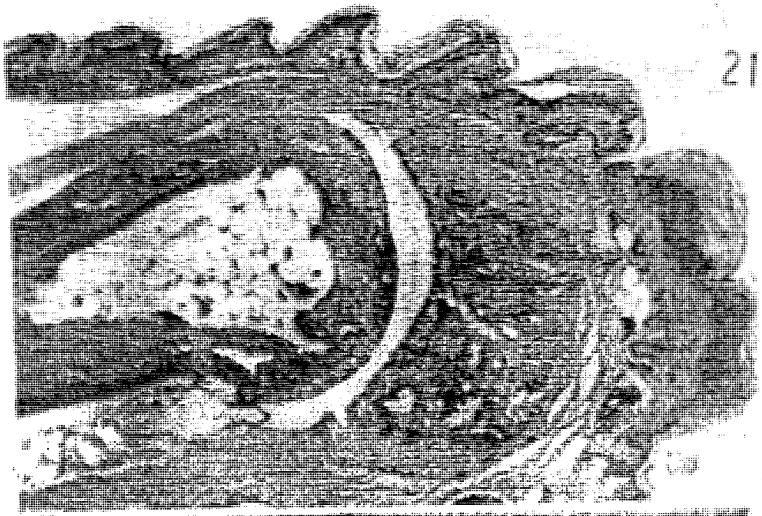


Fig. 1a

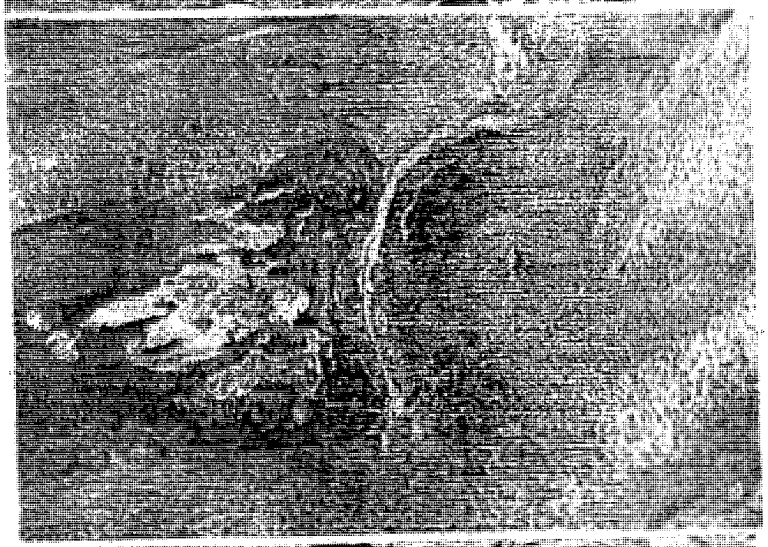


Fig. 1b



Fig. 1c

Samsung Bioepis Exhibit No. 1002
Page 8818

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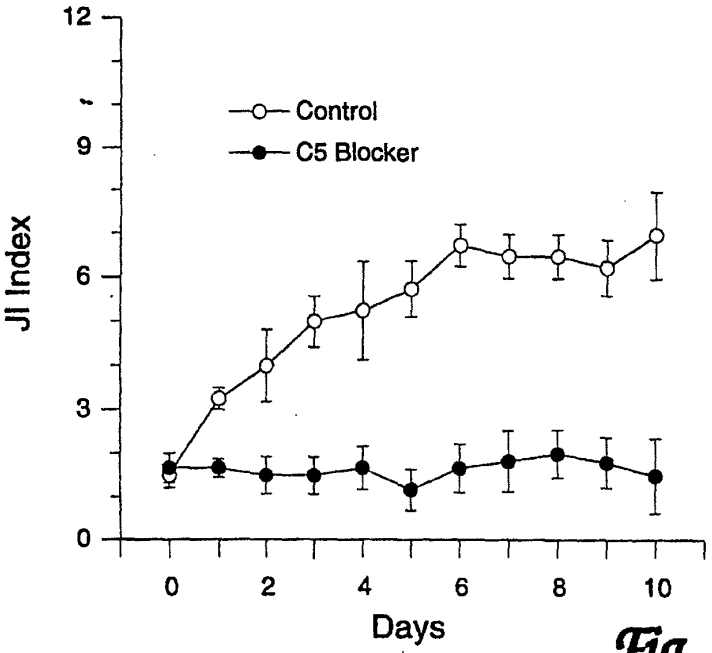


Fig. 2a

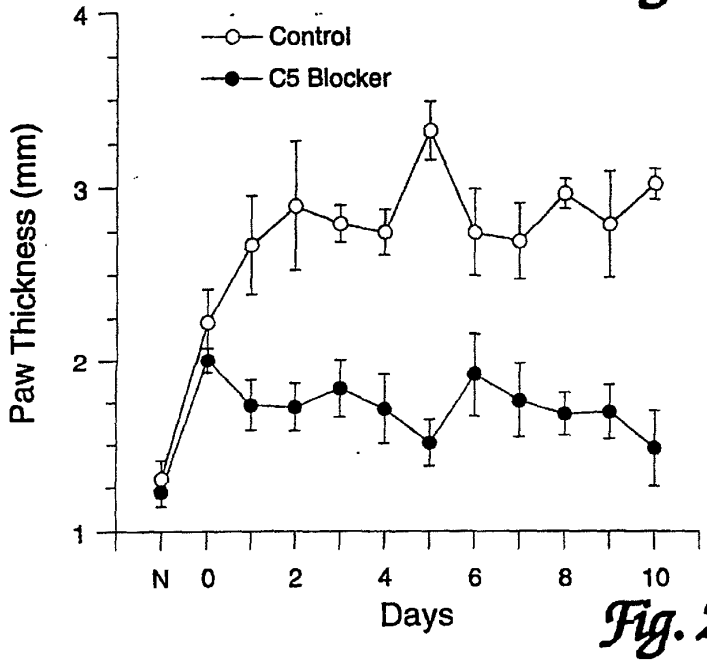


Fig. 2b

Samsung Bioepis Exhibit No. 1002
Page 8819

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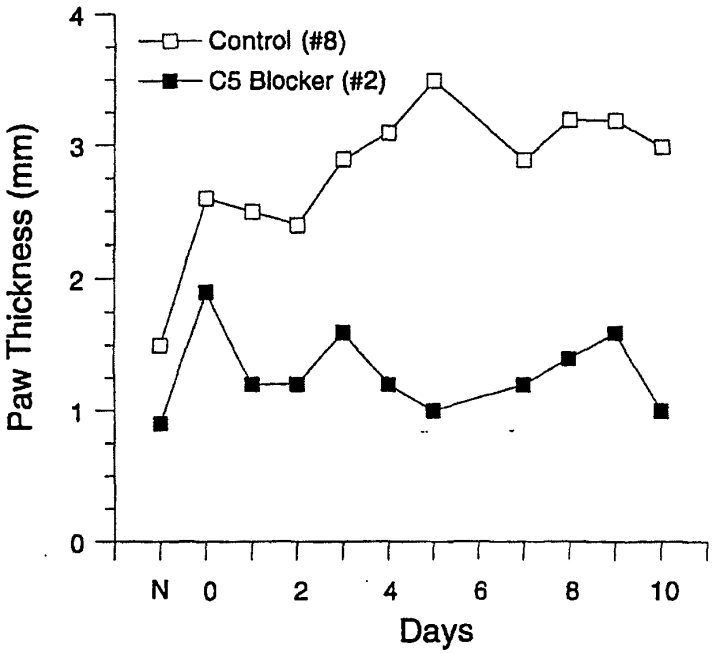


Fig. 3a

Samsung Bioepis Exhibit No. 1002
Page 8820

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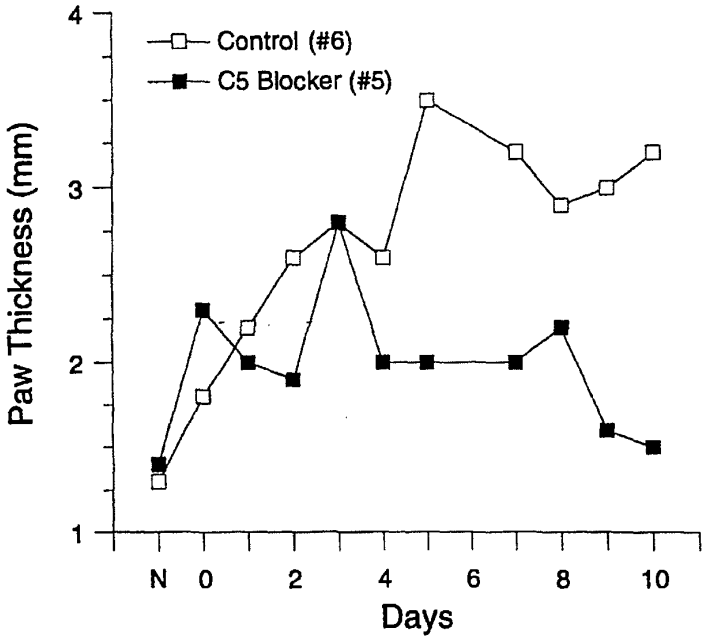


Fig. 3b

Samsung Bioepis Exhibit No. 1002
Page 8821

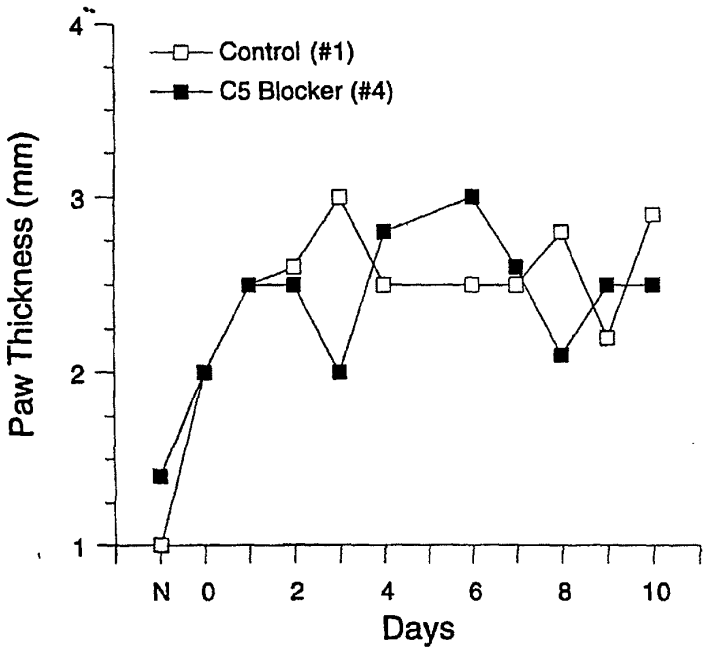


Fig. 3c

Samsung Bioepis Exhibit No. 1002
Page 8822

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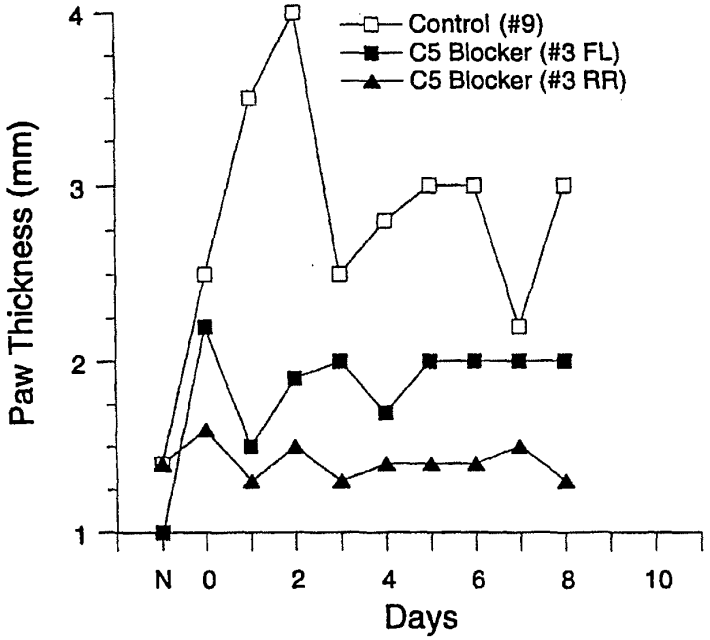


Fig. 3d

Samsung Bioepis Exhibit No. 1002
Page 8823

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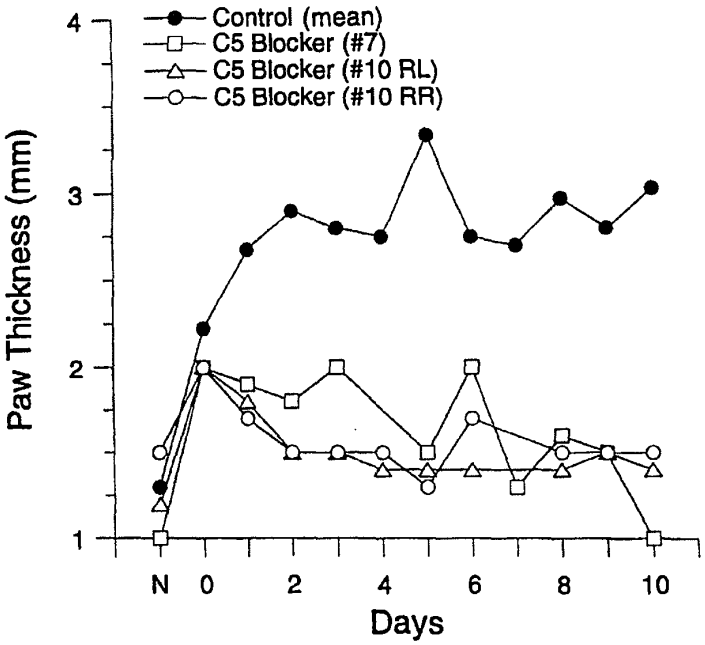


Fig. 3e

Samsung Bioepis Exhibit No. 1002
Page 8824

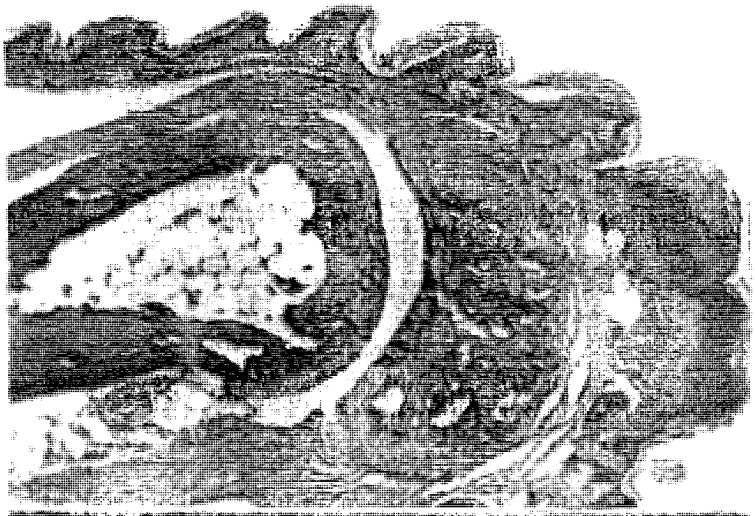


Fig. 4a



Fig. 4b



Fig. 4c

Samsung Bioepis Exhibit No. 1002
Page 8825

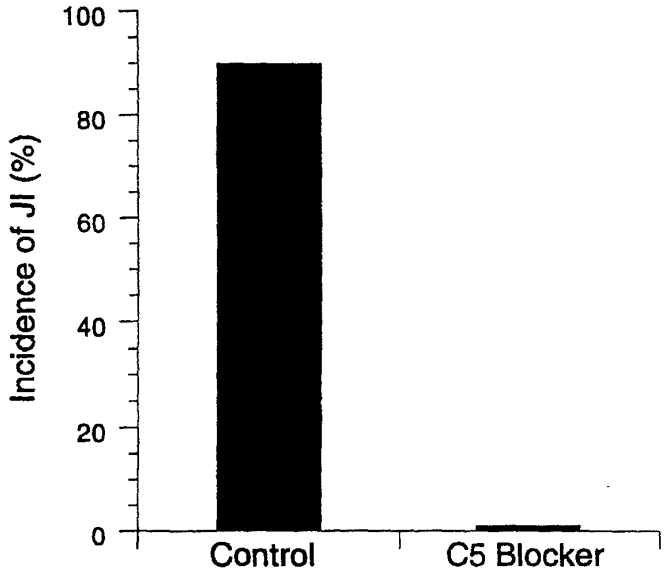


Fig. 5a

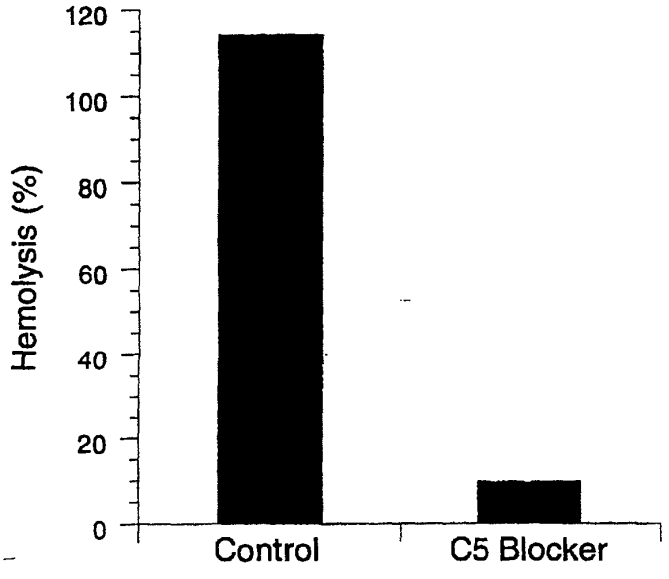


Fig. 5b

Samsung Bioepis Exhibit No. 1002
Page 8826

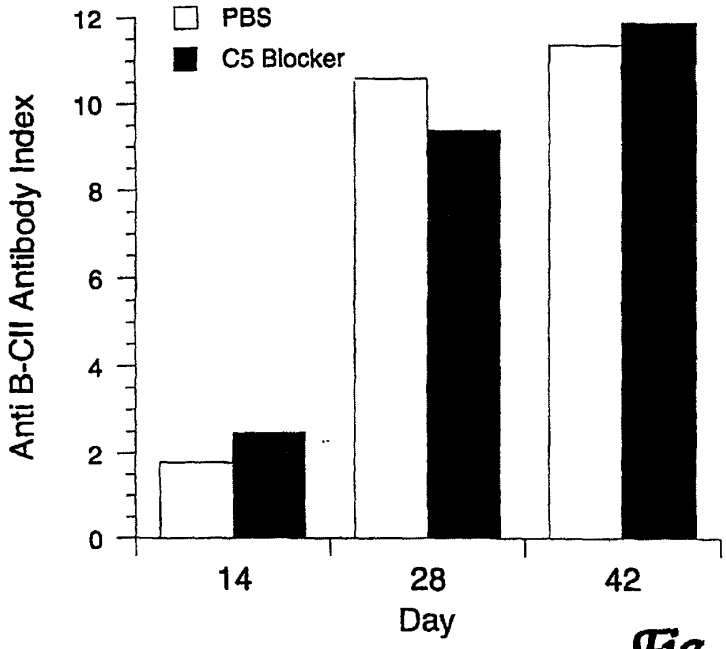


Fig. 6a

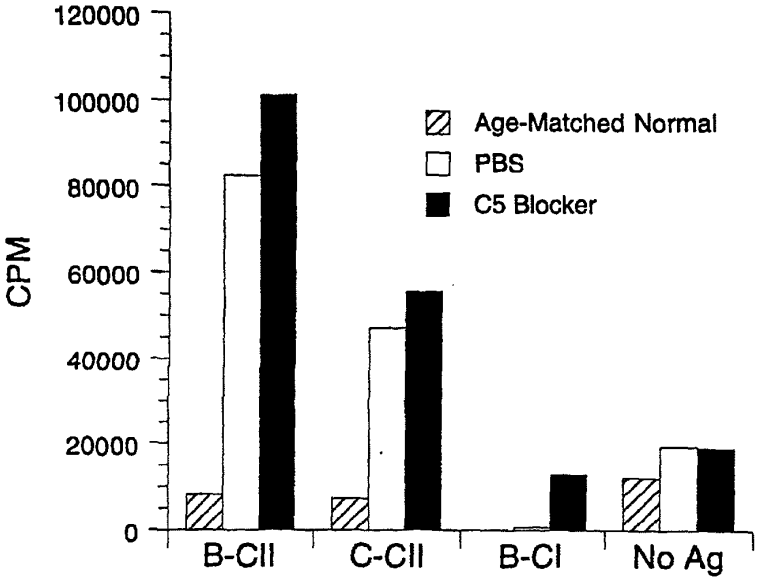


Fig. 6b

Samsung Bioepis Exhibit No. 1002
Page 8827

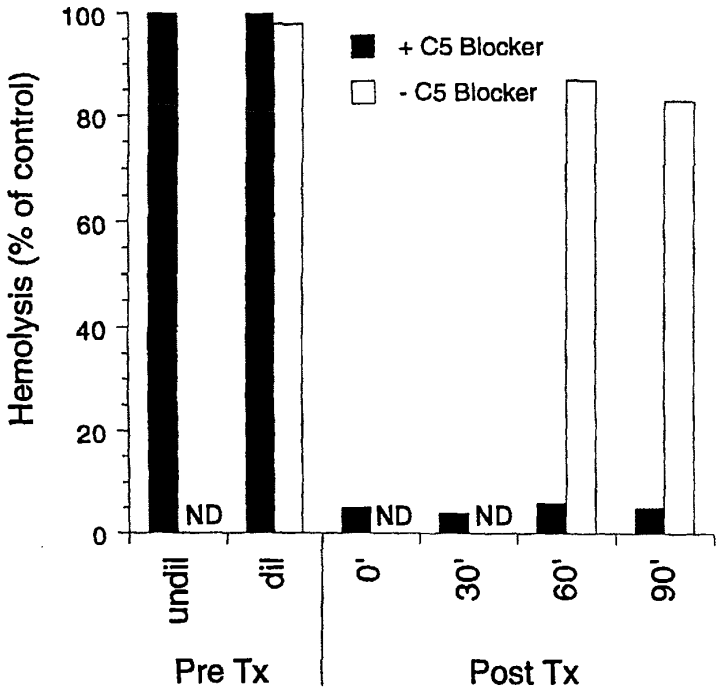


Fig. 7

Samsung Bioepis Exhibit No. 1002
Page 8828

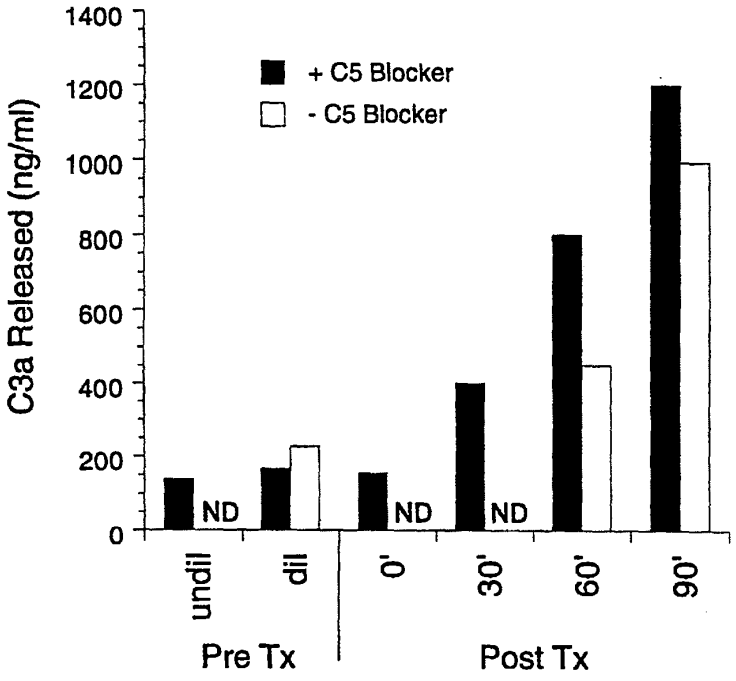

1400
1200
1000
800
600
400
200
0
C3a Released (ng/ml)
+ C5 Blocker
- C5 Blocker
ND
ND
ND
undil
dil
0'
30'
60'
90'
Pre Tx
Post Tx

Fig. 8

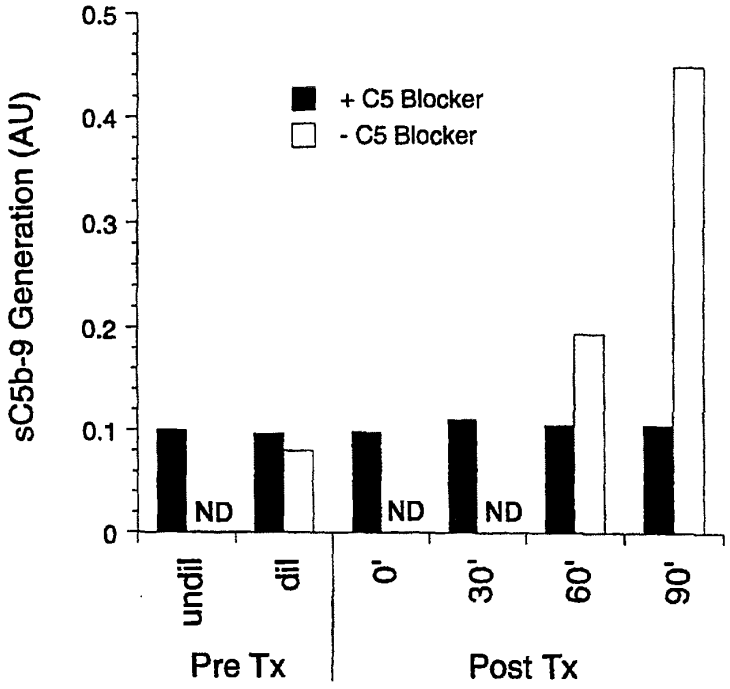


Fig. 9

Samsung Bioepis Exhibit No. 1002
Page 8830

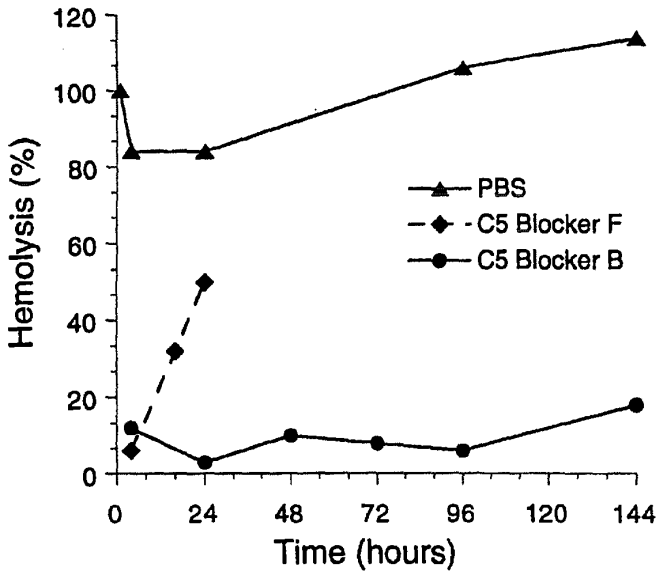


Fig. 10a

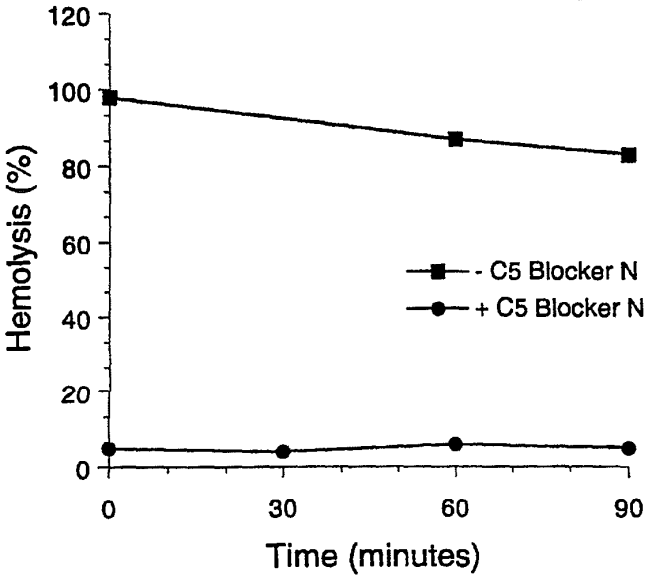


Fig. 10b

Electronic Acknowledgement Receipt	
EFS ID:	8435394
Application Number:	11127438
International Application Number:	
Confirmation Number:	5259
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Customer Number:	28120
Filer:	Xuqiong Wu/ginny blundell
Filer Authorized By:	Xuqiong Wu
Attorney Docket Number:	ALXN-P02-102
Receipt Date:	16-SEP-2010
Filing Date:	11-MAY-2005
Time Stamp:	17:40:34
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	no

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1	Transmittal Letter	ALXN_P02_102_Statement.pdf	93152 5d726bdb46e059fae08525c690b056d213db5a89	no	2
Warnings:					
Information:					

Samsung Bioepis Exhibit No. 1002
Page 8832

2	Information Disclosure Statement (IDS) Filed (SB/08)	ALXN_P02_102_SB08.pdf	125221 ab7d21a2f9ba198baf785d4400ffb1b40f6774a2a	no	2
Warnings:					
Information:					
This is not an USPTO supplied IDS fillable form					
3	Miscellaneous Incoming Letter	ALXN_P02_102_CA_OA.pdf	277818 2f2a45a23d17d8610e54a2b2f416a219d5e8268a	no	5
Warnings:					
Information:					
4	Foreign Reference	CA_2189015_Part1.pdf	3248610 e0fecaf8ea6071b9cd598e06476d707de6b9ad01	no	90
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		Total Files Size (in bytes):	8403506		

Samsung Bioepis Exhibit No. 1002
Page 8833

This Acknowledgement Receipt evidences receipt on the noted date by the USPTO of the indicated documents, characterized by the applicant, and including page counts, where applicable. It serves as evidence of receipt similar to a Post Card, as described in MPEP 503.

New Applications Under 35 U.S.C. 111

If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371

If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office

If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Samsung Bioepis Exhibit No. 1002
Page 8834

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Wang

Application No.: 11/127,438 Confirmation No.: 5259

Filed: May 11, 2005 Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES Examiner: Phillip Gambel

SUPPLEMENTAL INFORMATION DISCLOSURE STATEMENT (IDS)

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

In accordance with 37 CFR 1.97, Applicants hereby make of record the following additional documents. A PTO Form SB/08 and a full copy of each of the documents required under 37 CFR 1.98(a)(2) accompany this statement.

Applicants have become aware of the following documents, cited in a Canadian Official Action issued July 9, 2010, during the prosecution of Canadian Patent Application No. 2,496,834, which corresponds to the above referenced application, and in accordance with 37 CFR 1.97(c) and (e)(1) or (b)(3), hereby submit these documents for the Examiner's consideration. These documents are cited on the enclosed PTO Form SB/08, and a copy of the Canadian Official Action and of each document required under 37 CFR 1.98(a)(2) cited thereon are enclosed as well.

This statement is not to be interpreted as a representation that the cited documents are material, that an exhaustive search has been conducted, or that no other relevant information exists. Nor shall the citation of any document herein be construed *per se* as a representation that such

document is prior art. Moreover, Applicants understand the Examiner will make an independent evaluation of the cited documents.

This Supplemental Information Disclosure Statement is filed more than three months after the U.S. filing date, and after the mailing date of the first Office Action on the merits, whichever occurs first, but before the mailing date of a Final Office Action or Notice of Allowance (37 CFR 1.97(c)).

I hereby certify, pursuant to 37 CFR 1.97(e)(1), that each item of information contained in this Supplemental Information Disclosure Statement was first cited in any communication from a foreign patent office in a counterpart foreign application not more than three months prior to the filing of this Supplemental Information Disclosure Statement.

The Director is hereby authorized to charge any deficiency in the fees filed, asserted to be filed or which should have been filed herewith (or with any paper hereafter filed in this application by this firm) to our Deposit Account No. 18-1945, under Order No. ALXN-P02-102.

Dated: September 16, 2010

Respectfully submitted,

By___/Xuqiong Wu/___
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Attorneys/Agents For Applicant

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Wang

Application No.: 11/127,438 | Confirmation No.: 5259

Filed: May 11, 2005 | Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES | Examiner: P. Gambel

AMENDMENT IN RESPONSE TO NON-FINAL OFFICE ACTION UNDER 37 C.F.R. 1.111

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

INTRODUCTORY COMMENTS

In response to the Office Action dated August 17, 2010, Applicant petitions for a one-month extension of time with the required fee and submits this amendment and response. Please amend the above-identified U.S. patent application as follows:

Amendments to the specification begin on page 2 of this paper.

Amendments to the Claims are reflected in the listing of claims which begins on page 4 of this paper.

Remarks/Arguments begin on page 9 of this paper.

AMENDMENTS TO THE SPECIFICATION

Please replace paragraph [0014] with the following:

-- [0014] A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC® Jet Plus or Hudson T UP-DRAFT® II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., MicroAir® by Omron) and a shockwave nebulizer (Evit Labs Sonik LDI20). --

Please replace paragraph [0062] with the following:

-- [0062] Nebulizers produce a mist of drug-containing liquid droplets for inhalation. They are usually classified into two types: ultrasonic nebulizers and jet nebulizers. A new type of nebulizer is also available, which does not require ultrasound or air pressure to function. Single breath atomizers have also been developed (e.g., Respimat®), which is used to deliver a drug in a single inhalation and may be preferred because of less contamination. Jet nebulizers are more common and use a source of pressurized air to blast a stream of air through a drug-containing water reservoir, producing droplets in a complex process involving a viscosity-induced surface instability that leads to nonlinear phenomena in which surface tension and droplet breakup on baffles play a role. Ultrasonic nebulizers produce droplets by mechanical vibration of a plate or mesh. In either type of nebulizer, the drug is usually contained in solution in the liquid in the nebulizer and so the droplets being produced contain drug in solution. However, for some formulations (e.g., Pulmicort®) the drug is contained in small particles suspended in the water, which are then contained as particles suspended inside the droplets being produced. Certain excipients are usually included in formulations suitable for nebulization, such as sodium chloride (e.g., to maintain isotonicity), mineral acids and bases (e.g., to maintain or adjust pH), nitrogen headspace sparging, benzalkonium chloride, calcium chloride, sodium citrate, disodium edtate, and polysorbate 80. --

Please replace paragraph [0068] with the following:

-- [0068] An additional active agent (or an active agent in addition to the antibody therapeutic) of the present application can be another antibody therapeutic (e.g., an anti-complement or anti-C5 antibody, an anti-IgE antibody such as Xolair® or omalizumab, an anti-IL-4 antibody or an anti-IL-5 antibody), an anti-IgE inhibitor (e.g., Singulair® or montelukast sodium), a sympathomimetic (e.g., albuterol), an antibiotic (e.g., tobramycin), a deoxyribonuclease (e.g., Pulmozyme®), an anticholinergic drug (e.g., ipratropium bromide), a corticosteroid (e.g., dexamethasone), a β-adrenoreceptor agonist, a leukotriene inhibitor (e.g., zileuton), a 5 Lipoxygenase inhibitor, a PDE inhibitor, a CD23 antagonist, an IL-13 antagonist, a cytokine release inhibitor, a histamine H1 receptor antagonist, an anti-histamine, an anti-inflammatory agent (e.g. cromolyn sodium) or a histamine release inhibitor. As used herein, an active agent may also be referred to as a therapeutic or a drug. --

Please replace paragraph [0073] with the following:

-- [0073] A nebulizer of the present application can be a jet air nebulizer (e.g., Pari LC® Jet Plus or Hudson T UP-DRAFT® II), an ultrasonic nebulizer (e.g., MABISMist II), a vibrating mesh nebulizer (e.g., MicroAir® by Omron) and a shockwave nebulizer (Evit Labs Sonik LDI20). --

Please replace paragraph [0082] with the following:

-- [0082] In certain embodiments, an aerosol or nebulization antibody composition can be combined with one or more other aerosol or nebulization treatments, such as sympathomimetics (e.g., albuterol), antibiotics (e.g., tobramycin), deoxyribonucleases (e.g., Pulmozyme®), anticholinergic drugs (e.g., ipratropium bromide), or corticosteroids. --

AMENDMENTS TO THE CLAIMS

This listing of claims will replace all prior versions and listings of claims in the application:

1-14. (Cancelled)

15. (Currently amended) An aerosol composition comprising an anti-C5 antibody that inhibits ~~activation of the complement cascade,~~ the conversion of C5 into C5a and C5b, wherein the composition is suitable for treating a pulmonary condition in a subject.

16. (Original) The aerosol composition of claim 15, wherein the antibody is formulated in a composition suitable for aerosolization.

17. (Original) The aerosol composition of claim 15 further comprising an additional active agent.

18. (Currently amended) A biopharmaceutical package comprising: a) an anti-C5 antibody that inhibits ~~activation of the complement cascade;~~ the conversion of C5 into C5a and C5b; and b) a nebulizer, wherein the antibody is suitable for treating a pulmonary condition in a subject.

19. (Original) The biopharmaceutical package of claim 18, wherein the nebulizer is selected from the group consisting of a jet air nebulizer, an ultrasonic nebulizer, a vibrating mesh nebulizer and a shockwave nebulizer.

20. (Original) The biopharmaceutical package of claim 19 further comprising instructions for use.

21. (Original) The biopharmaceutical package of claim 19 further comprising an additional active agent.

22. (Currently amended) A pulmonary drug delivery kit comprising: a) an inhaler; and b) an anti-C5 antibody formulated for inhalation delivery, wherein said antibody inhibits ~~activation of the complement cascade~~ the conversion of C5 into C5a and C5b.

23. (Original) The kit of claim 22, wherein said inhaler is a metered dose inhaler.

24. (Previously presented) The kit of claim 23, wherein the metered dose inhaler is a pressurized metered dose inhaler.

25. (Original) The kit of any one of claims 22 or 23, wherein said inhaler further comprises a holding chamber.

26. (Original) The kit of claim 22, wherein said inhaler is a nebulizer.

27. (Original) The kit of claim 26, wherein said nebulizer is an ultrasonic nebulizer.

28. (Original) The kit of claim 26, wherein said nebulizer is a jet air nebulizer.

29. (Original) The kit of claim 22, wherein said inhaler is a dry powder inhaler.

30. (Canceled)

31. (Withdrawn) A method for preventing or treating a pulmonary condition in a subject, the method comprising administering to the subject an effective amount of the aerosol composition of claim 15 to prevent or treat the pulmonary condition.

32. (Withdrawn) The method of claim 31 wherein an additional active agent is administered.

33. (Withdrawn) The method of claim 31, wherein the pulmonary condition is selected from the group consisting of: asthma, bronchitis, a chronic obstructive pulmonary disease (COPD), interstitial lung diseases, lung malignancies, α-1 anti-trypsin deficiency, emphysema,

bronchiectasis, bronchiolitis obliterans, sarcoidosis, pulmonary fibrosis, and collagen vascular disorders.

34. (Withdrawn) The method of claim 31, wherein the subject is a human.

35-37. (Canceled)

38. (Withdrawn) The method of claim 31, wherein the antibody is eculizumab or pexelizumab.

39. (Withdrawn) The method of claim 31, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

40. (Withdrawn) The method of claim 39, wherein the mutated Fc portion does not activate complement.

41. (Withdrawn) The method of claim 31, wherein the antibody is administered prior to manifestation of the pulmonary condition, during manifestation of the pulmonary condition, or after manifestation of the pulmonary condition.

42. (Withdrawn) The method of claim 32, wherein the additional active agent is a corticosteroid.

43. (Withdrawn) The method of claim 42, wherein the corticosteroid is dexamethasone.

44. (Withdrawn) The method of claim 32, wherein the antibody and the additional active agent are administered to the subject by the same delivery method or route.

45. (Withdrawn) The method of claim 32, wherein the antibody and the additional active agent are administered to the subject by different delivery methods or routes.

46. (New) The composition of claim 15, wherein the pulmonary condition is asthma.

47. (New) The biopharmaceutical package of claim 18, wherein the pulmonary condition is asthma.

48. (New) The method of claim 31, wherein the pulmonary condition is asthma.

49. (New) The composition of claim 15, wherein the antibody is eculizumab or pexelizumab.

50. (New) The composition of claim 15, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

51. (New) The composition of claim 50, wherein the mutated Fc portion does not activate complement.

52. (New) The biopharmaceutical package of claim 18, wherein the antibody is eculizumab or pexelizumab.

53. (New) The biopharmaceutical package of claim 18, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

54. (New) The biopharmaceutical package of claim 53, wherein the mutated Fc portion does not activate complement.

55. (New) The pulmonary drug delivery kit of claim 22, wherein the antibody is eculizumab or pexelizumab.

56. (New) The pulmonary drug delivery kit of claim 22, wherein the antibody is selected from the group consisting of: a full length immunoglobulin, a single chain antibody, a domain antibody, a Fab fragment, and an antibody having a Fab fragment and a mutated Fc portion.

57. (New) The pulmonary drug delivery kit of claim 56, wherein the mutated Fc portion does not activate complement.

REMARKS

Claims 15-45 were pending. Claims 15, 18 and 22 have been amended, which amendments are supported by the application as filed, e.g., at page 13, lines 2 and 3 of U.S. Provisional Application No. 60/408,571 and paragraph [0011] of the instant application. Claims 30 and 35-37 have been canceled. Claims 46-57 have been added; the new claims are supported throughout the application (including the claims) as filed, e.g., Example 3 (page 17, line 16 to page 19, line 6; page 11, lines 13-20 of U.S. Provisional Application No. 60/408,571 and paragraph [0067] of the instant application. Accordingly, the claim amendments do not introduce any new matter.

Formal Amendments

The Office Action states that the title of the invention is not descriptive and requires that Applicant restrict the title to the claimed invention. Accordingly, Applicant would consider amending the title upon finding of allowable subject matter. Applicant has also amended the specification to identify trademarks.

Election of Invention

The Office Action states that claims 31-45 submitted on September 21, 2009 are directed to an invention that is independent or distinct from the invention originally claimed because claims 31-45 are drawn to methods of treating pulmonary conditions with the aerosol composition of the elected invention. While the claims are related as products and processes of use, the Office Action states that "the antibody in the aerosol composition can be used for affinity purification or immunodetection as well as other methods of treatment . . . [and the] methods of treating pulmonary conditions can be accomplished by numerous pharmaceutical agents other than anti-C5 antibodies." Applicants respectfully traverse.

Solely to expedite prosecution, Applicants have withdrawn the method claims (claims 31-34 and 38-45) from consideration. However, Applicants note that the method claims at issue depend from the aerosol composition claim (claim 15) and require all limitations of that claim.

Accordingly, upon allowance of the composition claim, rejoinder of the withdrawn methods claims will be requested.

Priority

The Office Action states that the "previous priority applications do not support sufficient written description for the instant claims." Specifically, the Office Action lists certain claim limitations as allegedly lacking sufficient written description in the priority applications. Applicants respectfully traverse and set forth below detailed support in the priority applications for each of the claim limitations.

As an initial matter, the instant claims find full written description support in the present application. The claims also find support in each of the priority applications: U.S. Provisional Application Nos. 60/408,571, filed on 6 September 2002, and 60/469,189, filed on 9 May 2003.

"The subject matter of [a] claim need not be described literally (using the same terms or *in haec verba*) in order for the disclosure to satisfy the written description requirement." MPEP § 2163.02. The MPEP also cites *Martin v. Johnson*, 424 F.2d 746, 751 (CCPA 1972) that "the description need not be *ipsis verbis* [i.e., in the same words] to be sufficient." MPEP § 2163 (3)(a). Moreover, "[m]ere rephrasing of a passage does not constitute new matter… [and] a rewording of a passage where the same meaning remains intact is permissible." MPEP § 2163.07 (I).

In accordance with these principles, the priority documents – and particularly Application No. 60/408,571 filed on 6 September 2002 – provide ample support for the terms "compositions / kits / packages", "antibody specificities", and "pulmonary conditions." For example, each of the priority documents states:

> Asthma, bronchitis and emphysema are known collectively as Chronic Obstructive Pulmonary Diseases…characterized as generalized airway obstruction, especially of the small airways, associated with varying degrees of symptoms of chronic bronchitis, asthma, and emphysema.

See, e.g., Application Nos. 60/408,571 and 60/469,189 both at page 1, lines 10 to 13. Notably, the above-quoted language is maintained *ipsis verbis* in the present specification. Paragraph [0002] of U.S. Patent Application Publication No. 2005/0271660.

The priority applications further state that the pulmonary diseases can also be associated with "airway hyper-responsiveness to specific and/or non-specific stimuli" and inflammation. Application No. 60/408,571 at page 1, lines 19 and 20, and page 3, lines 1 to 18. Thus one of ordinary skill in the art would easily and readily recognize that the priority applications describe a genus of "obstructive pulmonary disorders" sharing common clinical manifestations such as inflammation, airway hyper-responsiveness, and obstruction of, e.g., the small airways. It would have been equally clear to the artisan that the priority documents provide a representative number of species of that genus (such as, e.g., asthma, bronchitis, and emphysema) such that the artisan would understand that applicant was in full possession of the genus at the asserted earliest priority date of 6 September 2002.

Fully consistent with and supported by both priority documents, the present claims cover compositions suitable for treating "pulmonary conditions." Under the section entitled "Pulmonary Diseases or Conditions, and the Complement System" (page 4, paragraphs [0046] to [0057]), the instant specification states that "pulmonary diseases or conditions such as asthma can be characterized by a combination of chronic airway inflammation, airway obstruction and airway hyper-responsiveness." The specification further provides a number of species within the genus of pulmonary conditions, the species including, among others, asthma, bronchitis, and emphysema.

In view of the foregoing, it is clear that the skilled artisan would fully appreciate the unmistakable harmony between the terms "pulmonary diseases," as recited in the priority documents, and "pulmonary condition," as recited in the instant specification. That is, the two genus terms are characterized by the same clinical manifestations and comprise the same species. Any distinction between these two clearly synonymous terms is at most nothing more than a "mere rephrasing" without a difference. Thus, Applicant asserts that the genus term "pulmonary condition" is fully supported in the priority documents as filed.

The Office Action also states that the terms "'kits . packages' do not appear to be described prior to the instant application." Applicant respectfully disagrees and asserts that the priority applications provide ample written support for the claimed kits, biopharmaceutical packages, and compositions.

For example, the priority applications each describe that a "compound that inhibits production and/or activity of at least one complement component will generally be in an aerosol form with a suitable pharmaceutical carrier[.]" Application No. 60/408,571 at page 14, lines 12 to 14. *See also* U.S. 60/469,189 at page 18, lines 15 to 17. They further state that this "complement-inhibiting compound … can be administered via … aerosol[.]" Application No. 60/408,571 at page 4, lines 5 to 11. A skilled artisan at the filing date of the '571 provisional application would have appreciated that administering the described aerosol compositions requires something more than the composition itself – namely: a delivery device such as an inhaler or a nebulizer – just as the artisan would have appreciated that an intravenous form of a composition would require, e.g., a syringe useful for intravenous delivery. While an applicant may not rely on obviousness *per se* as evidence of written description, "[i]nformation which is well known in the art need not be described in detail in the specification." MPEP § 2163 (II)(A)(2). Thus, from this disclosure alone, it is clear that the artisan would have easily and readily appreciated that Applicant was in full possession of the instantly claimed kits, packages, and compositions.

Notwithstanding these considerations, the priority documents specifically exemplify the use of such an aerosol composition – namely, an anti-C5 antibody formulated for, and administered to mammals, by way of aerosol and nebulization. Application No. 60/408,571 at pages 17-18 (Example 3), and Fig. 4a. According to the MPEP, adequate written description may be shown where, as here, an actual reduction to practice of the claimed invention is fully described. MPEP § 2163 (3)(a). In view of the foregoing, Applicant asserts that the terms kits, biopharmaceutical packages, and compositions are fully supported in the priority applications as filed.

Finally, the priority applications also provide ample support for the claimed "antibody specificities." The instant claims are drawn to various compositions, kits, and packages comprising

an anti-C5 antibody that inhibits conversion of C5 into C5a and C5b. *Ipsis verbis* support for such an antibody is provided in both priority documents. *See* Application No. 60/408,571 at page 10, second full paragraph (stating that "[p]articularly useful compounds for use herein are antibodies that reduce … the conversion of complement component C5 into complement components C5a and C5b"). Nothing more is required under the law.

For at least the foregoing reasons, the priority applications clearly provide sufficient support under 35 U.S.C. § 112, first paragraph for the instant claims. Accordingly, the instant claims are entitled to an effective filing date of 6 September 2002, based upon the disclosure of Application No. 60/408,571.

Rejections

1. Claims 15-17 are rejected under 35 U.S.C. § 102(b) as being anticipated by Wang (WO2004/022096).

The cited PCT application was published on 18 March 2004; it also claims priority to U.S. Provisional Application Nos. 60/408,571 and 60/469,189, the same as the present application. As detailed above, Applicant asserts that the instant claims (including claims 15-17 at issue here) are entitled to an effective filing date of 6 September 2002. Accordingly, Applicant requests reconsideration and withdrawal of this rejection.

2. Claims 15-18, 22 and 30 are rejected under 35 U.S.C. § 102(b)(e) as being anticipated by Krause et al. (US 2004/0014782).

The Krause application was published on 22 January 2004 and claims priority to U.S. Provisional Application No. 60/368,925, filed on 29 March 2002. Accordingly, the Office Action cites Krause under 102(b)(e), as the instant claims are denied of the priority claim but may be entitled to the earlier priority dates.

First, for at least the reasons presented above, Applicant asserts that the instant claims are fully entitled to a priority date of 6 September 2002. Accordingly, Krause, assuming *arguendo* that it is relevant to the claims, applies, if at all, under § 102(e).

To demonstrate that the claimed invention had been conceived prior to the effective filing date of the Krause et al. reference, Applicant files concurrently herewith an executed Declaration by the inventor under 37 C.F.R. § 1.131. As detailed in the Declaration, the inventor had conceived of the invention by 20 December 2001 or earlier, as indicated by the dated experimental plans on evaluating the efficacy of anti-C5 antibody BB5.1 using an asthma mouse model. As demonstrated by Examples 1-3 of the priority application, No. 60/408,571 (filed on 6 September 2002), the inventor performed the animal studies as planned.

Thus the Declaration establishes that Applicant conceived of the invention prior to 29 March 2002, the filing date of the priority application of Krause and was diligent from prior to 29 March 2002 to at least 6 September 2002. Applicant further reserves the right to provide evidence of an even earlier date of conception. Accordingly, Krause cannot serve as a 102(e) reference against the instant application, and reconsideration and withdrawal of this rejection are respectfully requested.

Applicant's submission of the antedating Declaration is, however, in no way a concession to the reasons stated in the Office Action underlying the rejections based on Krause. Assuming *arguendo*, Krause were prior art against the instant application, Applicant provides the following remarks in support of patentability of the instant claims.

The instant claims are drawn to compositions (e.g., packages and kits) suitable for treating pulmonary conditions such as asthma in a subject. The claimed compositions, kits, and packages comprise an anti-C5 antibody that inhibits conversion of C5 into C5a and C5b.

Krause et al. discloses a genus of small molecule C5a receptor inhibitors for use in treating inflammation including disorders such as rheumatoid arthritis, skin injuries, infections, and lung diseases such as asthma. According to the Office Action, Krause et al. anticipates the instant claims

because it "teach[es] the use of C5a antagonists, including anti-C5 antibodies …in the treat[ment of] respiratory diseases [and] lung disorders, including ARDS and asthma." Applicant respectfully disagrees for at least two reasons: first, the C5a antagonists disclosed by Krause do not include anti-C5 antibodies; second, the reference does not disclose methods or kits including anti-C5 antibodies for use in treating respiratory disorders.

a. The "C5a Antagonists" of Krause do not include anti-C5 antibodies.

At paragraphs [0035] and [0036], Krause defines two types of active agents that can be used for treating inflammation in accordance with its disclosure: "C5a antagonists" (or "C5a receptor antagonists") and "C5a receptor-inactive therapeutic agents." The "C5a antagonist," according to the reference, is "any compound that exhibits C5a antagonist activity within the a [sic] C5a receptor-mediated chemotaxis, radioligand binding assay, or calcium mobilization assay as provided [in the reference]." Krause et al. at page 3, paragraph [0035]. The reference further provides examples of numerous small molecule C5a antagonists and clarifies the nature of the three assays which are used to determine if a compound meets the definition of "C5a antagonist." *Id.* at pages 4 to 17, paragraphs [0040] to [0198]. Notably, in the nearly 14 full pages of C5a antagonists disclosed in Krause, there is not one mention of an anti-C5 antibody being a member of this genus.

To the contrary, anti-C5 antibodies are recited but two times in Krause, each time narrowly under the heading of "C5a receptor-inactive therapeutic agent," and, as elaborated on below, only in conjunction with treating rheumatoid arthritis. *See, e.g.*, Krause at page 18, paragraphs [0206] and [0207] (stating that "at least one C5a receptor-inactive therapeutic agent is … an anti-C5 monoclonal antibody"). "C5a receptor-inactive therapeutic agents" are defined in the reference as any therapeutic agent that is not a C5a antagonist. *Id.* at page 4, paragraph [0036].

Thus, contrary to the Office Action's interpretation of the reference, Krause's genus of C5a antagonists does not include anti-C5 antibodies. In fact, the reference teachings – namely: that anti-C5 antibodies are not C5a antagonists – are actually diametrically opposite of the interpretation asserted by the Office Action.

b. The Krause asthma-related methods and kits do not include anti-C5 antibodies.

The second assertion by the Office Action is that Krause discloses methods and/or kits including anti-C5 antibodies for use in treating respiratory disorders. Applicant also respectfully disagrees.

In the section of its disclosure titled "Combination Therapy," Krause discloses in a distinct and modular way specific combination therapies useful for treating different types of inflammation. That is, in each therapeutic "module" related to the treatment of a specific inflammatory condition (e.g., rheumatoid arthritis), Krause provides a number of specific C5a receptor-inactive therapeutic agents that can be used in combination with the C5a receptor antagonists of its disclosure. For example, at pages 17 and 18, Krause discloses a combination therapy for treating rheumatoid arthritis, which therapy includes "at least one C5a antagonist and a C5a receptor-inactive agent that is an anti-arthritic agent (i.e., a C5a receptor-inactive anti-arthritic agent)." *Id.* at page 17, paragraph [0202] (emphasis added). In another therapeutic module, Krause provides a combination therapy for treating lung disorders using at least one C5a antagonist and a C5a receptor-inactive agent "useful in the treatment of asthma." *Id.* at page 18, paragraph [0227] and 23, paragraph [0279].

Only twice are anti-C5 antibodies (at paragraphs [0207] and [0277]) recited in Krause, and in each instance only in "rheumatoid arthritis" modules and only as one of a laundry list of well-known, potential C5a receptor-inactive anti-arthritic agents such as, e.g., NSAIDs, COX inhibitors, anti-TNF agents, anti-IL-1 agents, and anti-CD20 agents, useful for treating rheumatoid arthritis. Notably, at the effective date of Krause, Alexion Pharmaceuticals, Inc. was engaged in a well-known phase II clinical trial evaluating an anti-C5 antibody in rheumatoid arthritis. *See, e.g.*, PR Newswire article dated 4 August 1999 and Abstract of Kaplan (2002) Curr Opin Investig Drugs 3(7):1017-1023 (stating that - "Eculizumab … is under development by Alexion as a potential treatment for … rheumatoid arthritis (RA) … In January 2002, a phase IIb trial was initiated for RA").

In contrast, the two therapeutic modules drawn to treating lung disorders – at pages 18, 19, and 23 of Krause – are devoid of any mention of anti-C5 antibodies, let alone as being one of the C5a receptor-inactive agents useful for treating asthma. Instead, the lung disorder-related therapeutic modules recite a lung disorder-specific laundry list of well-known asthma treatments including, e.g., anti-thrombin agents, beta adrenergic receptor agonists, and corticosteroids. Krause et al. at pages 18-19, paragraphs [0227] to [0229]. To be clear: there is no disclosure or even a suggestion in Krause of the use of an anti-C5 antibody, either alone or in combination with a C5a antagonist, for the treatment of a lung disorder such as asthma, despite the fact that anti-C5 antibodies had been known in the art. Rather, as discussed above, Krause teaches that an anti-C5 antibody is not a C5a antagonist and does not include such antibodies, even as a supplemental agent, in its asthma-related therapeutic modules.

In view of the foregoing, it is clear that anti-C5 antibodies are not part of the genus of C5a antagonists recited in Krause. Moreover, it is clear that neither the asthma-related methods nor kits disclosed in Krause feature an anti-C5 antibody, as instantly claimed. Accordingly, the instant claims are not anticipated by Krause and Applicant respectfully requests reconsideration and withdrawal of the rejection under 35 U.S.C. § 102.

3. Claims 15-30 are rejected under 35 U.S.C. § 103(a) as being unpatentable over Krause in view of West et al. (U.S. Patent No. 7,071,299), Babington (U.S. Patent No. 4,228,795) and Lobb et al (U.S. Patent No. 5,871,734).

Applicant maintains that Krause is not properly 102(e) prior art against the instant application. In the alternative, assuming *arguendo*, Krause could serve as a prior art against the instant application, Applicant maintains that Krause does not teach or suggest an anti-C5 antibody in asthma-related methods and compositions. The secondary references are directed to methods and compositions suitable for nasal inhalation, but none of them cures the deficiency of Krause vis-a-vis the instant claims. Accordingly, Applicant respectfully requests reconsideration and withdrawal of this rejection.

In view of the above amendment, Applicant believes that the pending application is in condition for allowance.

Applicant petitions for one-month extension of time to file this amendment and response and submits the required fee herewith. If any additional fee is due, please charge our Deposit Account No. 18-1945, under Order No. ALXN-P02-102 from which the undersigned is authorized to draw.

Dated: December 17, 2010

Respectfully submitted,

By__/Xuqiong Wu/_____
Xuqiong Wu, Ph.D., J.D.
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Attorneys/Agents For Applicant

AMENDMENT TRANSMITTAL LETTER			Docket No. ALXN-P02-102
Application No. 11/127,438	Filing Date May 11, 2005	Examiner P. Gambel	Art Unit 1644

Applicant(s): Wang et al.

Invention: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES

TO THE COMMISSIONER FOR PATENTS

Transmitted herewith is an amendment in the above-identified application.

The fee has been calculated and is transmitted as shown below.

CLAIMS AS AMENDED						
	Claims Remaining After Amendment	Highest Number Previously Paid		Number Extra Claims Present	Rate	
Total Claims	40	- 32	=	8	x 52.00	416.00
Independent Claims	3	- 5	=	0	x 220.00	0.00
Multiple Dependent Claims (check if applicable) ☐						
Other fee (please specify): Extension for response within first month						130.00
TOTAL ADDITIONAL FEE FOR THIS AMENDMENT:						546.00

☒ Large Entity ☐ Small Entity

☐ No additional fee is required for this amendment.

☒ Please charge Deposit Account No. 18-1945 in the amount of $ 546.00 .

☐ A check in the amount of $ _____ to cover the filing fee is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☒ The Director is hereby authorized to charge and credit Deposit Account No. 18-1945 as described below. s

☒ Credit any overpayment.

☒ Charge any additional filing or application processing fees required under 37 CFR 1.16 and 1.17.

/Xuqiong Wu/ Dated: December 17, 2010

Xuqiong Wu, Ph.D., J.D.
Attorney/Agent Reg. No.: 55,745

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PTO/SB/22 (07-09)
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PETITION FOR EXTENSION OF TIME UNDER 37 CFR 1.136(a) FY 2009 *(Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).)*	Docket Number (Optional) ALXN-P02-102
Application Number 11/127,438	Filed May 11, 2005
For NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES	
Art Unit 1644	Examiner P. Gambel

This is a request under the provisions of 37 CFR 1.136(a) to extend the period for filing a reply in the above identified application.

The requested extension and fee are as follows (check time period desired and enter the appropriate fee below):

		Fee	Small Entity Fee	
[x]	One month (37 CFR 1.17(a)(1))	$130	$65	$ 130.00
[ ]	Two months (37 CFR 1.17(a)(2))	$490	$245	$
[ ]	Three months (37 CFR 1.17(a)(3))	$1110	$555	$
[ ]	Four months (37 CFR 1.17(a)(4))	$1730	$865	$
[ ]	Five months (37 CFR 1.17(a)(5))	$2350	$1175	$

[ ] Applicant claims small entity status. See 37 CFR 1.27.

[ ] A check in the amount of the fee is enclosed.

[ ] Payment by credit card. Form PTO-2038 is attached.

[x] The Director has already been authorized to charge fees in this application to a Deposit Account.

[x] The Director is hereby authorized to charge any fees which may be required, or credit any overpayment, to Deposit Account Number 18-1945 .

WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.

I am the

[ ] applicant/inventor.

[ ] assignee of record of the entire interest. See 37 CFR 3.71.
Statement under 37 CFR 3.73(b) is enclosed. (Form PTO/SB/96).

[x] attorney or agent of record. Registration Number 55,745

[ ] attorney or agent under 37 CFR 1.34.
Registration number if acting under 37 CFR 1.34 _____

/Xuqiong Wu/	December 17, 2010
Signature	Date
Xuqiong Wu, Ph.D., J.D.	(617) 951-7027
Typed or printed name	Telephone Number

NOTE: Signatures of all the inventors or assignees of record of the entire interest or their representative(s) are required. Submit multiple forms if more than one signature is required, see below.

[x] Total of 1 forms are submitted.

Docket No.: ALXN-P02-102
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:
Wang

Application No.: 11/127,438 | Confirmation No.: 5259

Filed: May 11, 2005 | Art Unit: 1644

For: NEBULIZATION OF MONOCLONAL ANTIBODIES FOR TREATING PULMONARY DISEASES | Examiner: P. Gambel

DECLARATION UNDER 37 CFR § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, YI WANG, hereby declare and state as follows:

1. I am the named inventor of the patent application identified above, which teaches and claims, among other things, methods and compositions for preventing or treating a pulmonary condition in a subject.

2. I understand that the Examiner has rejected the pending claims in view of Krause et al. (U.S. Patent Application Publication No. 2004/0014782, published on January 22, 2004). The Krause et al. application was filed on March 27, 2003, and claims priority to U.S. Provisional Application No. 60/368,925, filed March 29, 2002. Accordingly, it is my understanding that the effective filing date of the Krause et al. reference is March 29, 2002.

3. Prior to March 29, 2002, I had conceived of the invention as described and claimed in the subject application in this country. In support of this, I attach as Exhibits A and B C documents prepared before March 29, 2002, which demonstrate that I had designed and prepared a mouse model to evaluate the efficacy of an anti-C5 antibody on asthma, a pulmonary condition.

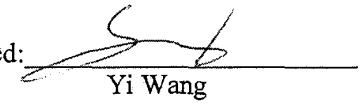
4. Exhibit A is a copy of my laboratory notebook page (Notebook #1060, page 45). This page was dated December 20, 2001, and also read and understood by my colleagues on at least two

separate occasions, one on March 12, 2002. This page detailed the experimental plans for evaluating the efficacy of anti-C5 antibody BB5.1 using an asthma mouse model.

5. Exhibit B is a copy of my laboratory notebook pages (Notebook #1060, pages 46 and 47). These pages were also read and understood by my colleagues on at least two separate occasions, one on March 14, 2002. These pages recorded the planned timeline for each mouse included in the study following the protocol detailed in the page shown as Exhibit A. The timeline starts with the date of birth of the mouse, date of sensitization, dates of air challenges, followed by various treatment (with the anti-C5 antibody or controls) dates. These laboratory notebook pages clearly demonstrate that I had conceived of the invention of using anti-C5 antibody in treating a pulmonary condition such as asthma well before March 29, 2002, the effective filing date of Krause et al.

6. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements are made with knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title XVIII of the United States Code and that willful false statements may jeopardize the validity of this Application for Patent or any patent issuing thereon.

Date: Dec. 16, 2010

Signed: 

Yi Wang

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Samsung Bioepis Exhibit No. 1002
Page 8858

PROJECT Asthma Continued from Page

12/20/01
Experimental plans for asthma study:

Purpose
Evaluate the efficacy of anti-C5 (BB5.1) on asthma mouse model.

Animals
BALB/c mice (8-12 weeks old) are purchased from the Jackson laboratory and maintained at Alexion's pathogen free facility.

Antigen sensitization and challenge
BALB/c mice will be sensitized on day 1 and 14 by intraperitoneal injection of 20ug OVA, (Grade V, Sigma) emulsified in 2mg aluminum hydroxide(Alum imject, Pierce) in a total volume of 100 ul.
All mice will be challenged daily with OVA for 10minutes via the airways (1% OVA in saline) for 3 days (day 28,29,and 30), using ultrasonic nebulization. In day 32, mice will be provoked with OVA (5% in saline) for 10 minutes to elicit an antigen-induced early-phase reaction (EPR) and late-phase reaction (LPR). Negative control mice will be sensitized with alum only and challenged with saline instead of OVA.

1 Plan one:
(1) prophylactic treatment
Beginning on day 25 the experimental groups will be injected with either anti-C5 (40mg/kg, S.C. injection in 0.2 ml saline) or Dex(2mg/kg, S.C. injection in 0.2 ml saline) also day 29 and 31.
After 5 hours of 5% OVA provocation, mice will be sacrificed and broncboalveolar lavage (BAL) will be performed by instilling of 1ml ice-cold PBS through the tracheal cannula, followed by gentle aspiration.

The BAL fluid will be collected.
1. Cell differential counts.
2. IgE measurement.
3. Analysis of cytokines Cell differential counts. IL-4,IL-5,IL-13 and TGF-beta
After BAL, lungs will be inflated with 10% buffered formalin(1ml), removed from chest cavityand fixed in 10% formalin at least 24h than stained with hematoxylin and eosin and examined by light microscopy for evaluation of the severity of inflammation.

(2) therapeutic treatment
In day 35, mice will be provoked with 5% OVA again, than the mice will be injected with anti-C5 (40mg/kg, I.V. injection in 0.2 ml saline) or Dex(2mg/kg, I.V. injection in 0.2 ml saline). After 5 hours, the mice will be done at both BAL and histology as the same as above.

2 Plan two:
In day 35, Baseline lung resistance and methacholine induced AHR will be measured in OVA sensitised mice and non-sensitised mice. By increasing doses of intravenous methacholine (5,15,45,137,411ug/kg), we will make invasive measurement of tracheal pressure in 5-min interval (see detail in our invasive method). Mice can be treated with BB5.1 in a dose of 40mg/kg 24 hours, 8 hours, 4 hours, and 2 hours before methacholine challenge. Control group will treated with 135.8 and PBS.

Continued on Page

Read and Understood By

Signed 3/12/02 Date Signed 9/25/02 Date

Samsung Bioepis Exhibit No. 1002
Page 8859

Sensitization BALB/c mice. exp1.

BALB/cJ From Jackson Laboratory.

BALB/c	DOB	Weight(g)	Route of administration	Dose of sensitization	Alum	Systimic time	Systimic time
386	11/26/01	23.8	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
387	11/26/01	24.4	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
388	11/26/01	25	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
389	11/26/01	27.5	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
390	11/26/01	24.8	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
391	11/26/01	22.7	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
392	11/26/01	24.1	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
393	11/26/01	22.7	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
394	11/26/01	24.8	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
395*	11/26/01	26.4	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
396	11/26/01	25.8	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
397	11/26/01	23.3	i.p.	20ug OVA	2mg	2/18/02(day1)	3/4/02(day14)
398C	11/26/01	22.4	i.p.	PBS	2mg	2/18/02(day1)	3/4/02(day14)
399C	11/26/01	26.1	i.p.	PBS	2mg	2/18/02(day1)	3/4/02(day14)
400C	11/26/01	26.4	i.p.	PBS	2mg	2/18/02(day1)	3/4/02(day14)

Airway challenge dose	Airway challenge time	Airway challenge time	Airway challenge time
1% OVA 8min	*3/18/02(day28)*	*3/19/02(day29)*	*3/20/02(day30)*
1% OVA 8min	*3/18/02(day28)*	*3/19/02(day29)*	*3/20/02(day30)*
1% OVA 8min	3/19/02(day28)	3/20/02(day29)	3/21/02(day30)
1% OVA 8min	*3/18/02(day28)*	*3/19/02(day29)*	*3/20/02(day30)*
1% OVA 8min	*3/18/02(day28)*	*3/19/02(day29)*	*3/20/02(day30)*
1% OVA 8min	3/19/02(day28)	3/20/02(day29)	3/21/02(day30)
1% OVA 8min	*3/18/02(day28)*	*3/19/02(day29)*	*3/20/02(day30)*
1% OVA 8min	3/19/02(day28)	3/20/02(day29)	3/21/02(day30)
1% OVA 8min	3/19/02(day28)	3/20/02(day29)	3/21/02(day30)
1% OVA 8min			
1% OVA 8min			
1% OVA 8min			
PBS 8min	*3/18/02(day28)*	*3/19/02(day29)*	*3/20/02(day30)*
PBS 8min	3/19/02(day28)	3/20/02(day29)	3/21/02(day30)
PBS 8min	3/19/02(day28)	3/20/02(day29)	3/21/02(day30)

Prepare OVA solution:

320ml OVA
704ml Alum
544 ml PBS

Control

270ml PBS
220ml Alum

Stir 30min

No 22161
lot# AE40391

Continued on Page

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Signed 3/14/02 Date

Signed 9/25/02 Date

Samsung Bioepis Exhibit No. 1002
Page 8860

PROJECT Asthma Study. Continued from Page

Schedule of experiment 1

2/16/02 Monday day1

3/4/02 Monday day14

3/15/02 Friday day25 Treatment group1 anti-C5, dex control antibody 6mice

3/16/02 Saturday day26 Treatment group2 anti-C5, dex control antibody 6 mice

3/18/02 Monday day28 challgene 1%OVA ¡Ś5min group1 measure 15min & 4h

3/19/02 Tuesday day29 challgene 1%OVA ¡Ś6 min & Treat group1

challgene 1%OVA ¡Ś6 min group2 measure 15min & 4h

3/20/02 Wednesday day30 challgene 1%OVA ¡Ś6 min group1 measure 15min &4h

Treat group2

3/21/02 Thursday day31 challgene 1%OVA ¡Ś5 min group2 measure 15min &4h

3/22/02 Friday day32 Treat group1

3/23/02 Saturday day33 treat group2

3/25/02 Monday challgene 1%OVA ¡Ś6 min group1 measure 15min &4h BAL

3/26/02 Tuesday challgene 1%OVA ¡Ś6 min group2 measure 15min &4h BAL

BALB/c	DOB	Weight(g)	Route of administration	Dose of sensitization	Alum	Systimic time	Systimic time
431	11/26/01	28.1	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
432	11/26/01	26.8	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
433	11/26/01	27.3	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
434	11/26/01	27.3	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
436	11/26/01	26.9	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
437	11/26/01	25.4	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
438	11/26/01	24.5	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
439	11/26/01	29.1	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
440	11/26/01	27.2	i.p.	20ug OVA	2mg	3/4/02(day1)	3/18/02(day14)
443	11/26/01	28.8	i.p.	PBS	2mg	3/4/02(day1)	3/18/02(day14)
444	11/26/01	27.6	i.p.	PBS	2mg	3/4/02(day1)	3/18/02(day14)
445	11/26/01	26.1	i.p.	PBS	2mg	3/4/02(day1)	3/18/02(day14)

Continued on Page

Read and Understood By

Signed — Date 3/14/02. Signed — Date 9/25/02

Samsung Bioepis Exhibit No. 1002
Page 8861

Electronic Patent Application Fee Transmittal

Application Number:	11127438
Filing Date:	11-May-2005
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Filer:	Xuqiong Wu/ginny blundell
Attorney Docket Number:	ALXN-P02-102

Filed as Large Entity

Utility under 35 USC 111(a) Filing Fees

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Basic Filing:				
Pages:				
Claims:				
Claims in excess of 20	1202	8	52	416
Miscellaneous-Filing:				
Petition:				
Patent-Appeals-and-Interference:				
Post-Allowance-and-Post-Issuance:				
Extension-of-Time:				

Samsung Bioepis Exhibit No. 1002
Page 8862

Description	Fee Code	Quantity	Amount	Sub-Total in USD($)
Extension - 1 month with $0 paid	1251	1	130	130
Miscellaneous:				
		Total in USD ($)		**546**

Samsung Bioepis Exhibit No. 1002
Page 8863

Electronic Acknowledgement Receipt	
EFS ID:	9061196
Application Number:	11127438
International Application Number:	
Confirmation Number:	5259
Title of Invention:	Nebulization of monoclonal antibodies for treating pulmonary diseases
First Named Inventor/Applicant Name:	Yi Wang
Customer Number:	28120
Filer:	Xuqiong Wu/ginny blundell
Filer Authorized By:	Xuqiong Wu
Attorney Docket Number:	ALXN-P02-102
Receipt Date:	17-DEC-2010
Filing Date:	11-MAY-2005
Time Stamp:	14:16:13
Application Type:	Utility under 35 USC 111(a)

Payment information:

Submitted with Payment	yes
Payment Type	Deposit Account
Payment was successfully received in RAM	$546
RAM confirmation Number	480
Deposit Account	181945
Authorized User	
The Director of the USPTO is hereby authorized to charge indicated fees and credit any overpayment as follows: Charge any Additional Fees required under 37 C.F.R. Section 1.17 (Patent application and reexamination processing fees) Charge any Additional Fees required under 37 C.F.R. Section 1.19 (Document supply fees)	

Samsung Bioepis Exhibit No. 1002
Page 8864

Charge any Additional Fees required under 37 C.F.R. Section 1.21 (Miscellaneous fees and charges)

File Listing:

Document Number	Document Description	File Name	File Size(Bytes)/ Message Digest	Multi Part /.zip	Pages (if appl.)
1		ALXN-P02-102_Reply.pdf	167366 4d45fa63b1d2299a453b7624d3276338c19f94d8	yes	18

Multipart Description/PDF files in .zip description

Document Description	Start	End
Amendment/Req. Reconsideration-After Non-Final Reject	1	1
Specification	2	3
Claims	4	8
Applicant Arguments/Remarks Made in an Amendment	9	18

Warnings:					
Information:					
2	Miscellaneous Incoming Letter	ALXN-P02-102_Transmittal.pdf	92368 fedf9b5ea37385fdb01068d620a031bfd18f5a3c	no	1
Warnings:					
Information:					
3	Extension of Time	ALXN-P02-102_EOT.pdf	106393 f6e259542a579450c238483d3bd629d15f57907a	no	1
Warnings:					
Information:					
4	Rule 130, 131 or 132 Affidavits	ALXN-P02-102_Declaration.pdf	88924 9894a2688738e1b7e9aaa1bcad76f3549c42b129	no	2
Warnings:					
Information:					
5	Miscellaneous Incoming Letter	Exhibit_A.pdf	321363 9b5aaf19ffa2713d059dc3762dbee9f5539e2f29	no	1
Warnings:					
Information:					
6	Miscellaneous Incoming Letter	Exhibit_B.pdf	622614 89a0b3a48ce18b3ad19575b33a494c1a8c9f0b8c	no	2
Warnings:					

Samsung Bioepis Exhibit No. 1002
Page 8865

Information:					
7	Fee Worksheet (PTO-875)	fee-info.pdf	31953 bafa62dc061e6f9d6d15dab337fa323c39b40f0b6	no	2
Warnings:					
Information:					
			Total Files Size (in bytes):	1430981	

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If a new application is being filed and the application includes the necessary components for a filing date (see 37 CFR 1.53(b)-(d) and MPEP 506), a Filing Receipt (37 CFR 1.54) will be issued in due course and the date shown on this Acknowledgement Receipt will establish the filing date of the application.

National Stage of an International Application under 35 U.S.C. 371
If a timely submission to enter the national stage of an international application is compliant with the conditions of 35 U.S.C. 371 and other applicable requirements a Form PCT/DO/EO/903 indicating acceptance of the application as a national stage submission under 35 U.S.C. 371 will be issued in addition to the Filing Receipt, in due course.

New International Application Filed with the USPTO as a Receiving Office
If a new international application is being filed and the international application includes the necessary components for an international filing date (see PCT Article 11 and MPEP 1810), a Notification of the International Application Number and of the International Filing Date (Form PCT/RO/105) will be issued in due course, subject to prescriptions concerning national security, and the date shown on this Acknowledgement Receipt will establish the international filing date of the application.

Samsung Bioepis Exhibit No. 1002
Page 8866

PTO/SB/06 (07-06)
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PATENT APPLICATION FEE DETERMINATION RECORD Substitute for Form PTO-875	Application or Docket Number 11/127,438	Filing Date 05/11/2005	☒ To be Mailed

APPLICATION AS FILED – PART I

	(Column 1)	(Column 2)	SMALL ENTITY ☒		OR	OTHER THAN SMALL ENTITY	
FOR	NUMBER FILED	NUMBER EXTRA	RATE ($)	FEE ($)		RATE ($)	FEE ($)
☐ BASIC FEE (37 CFR 1.16(a), (b), or (c))	N/A	N/A	N/A			N/A	
☐ SEARCH FEE (37 CFR 1.16(k), (l), or (m))	N/A	N/A	N/A			N/A	
☐ EXAMINATION FEE (37 CFR 1.16(o), (p), or (q))	N/A	N/A	N/A			N/A	
TOTAL CLAIMS (37 CFR 1.16(i))	minus 20 =	*	X $ =		OR	X $ =	
INDEPENDENT CLAIMS (37 CFR 1.16(h))	minus 3 =	*	X $ =			X $ =	
☐ APPLICATION SIZE FEE (37 CFR 1.16(s))	If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).						
☐ MULTIPLE DEPENDENT CLAIM PRESENT (37 CFR 1.16(j))							
* If the difference in column 1 is less than zero, enter "0" in column 2.			TOTAL			TOTAL	

APPLICATION AS AMENDED – PART II

		(Column 1)		(Column 2)	(Column 3)	SMALL ENTITY		OR	OTHER THAN SMALL ENTITY	
	12/17/2010	CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	* 40	Minus	** 32	= 8	X $26 =	208	OR	X $ =	
	Independent (37 CFR 1.16(h))	* 3	Minus	***5	= 0	X $110 =	0	OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE	208	OR	TOTAL ADD'L FEE	

		(Column 1)		(Column 2)	(Column 3)					
		CLAIMS REMAINING AFTER AMENDMENT		HIGHEST NUMBER PREVIOUSLY PAID FOR	PRESENT EXTRA	RATE ($)	ADDITIONAL FEE ($)		RATE ($)	ADDITIONAL FEE ($)
AMENDMENT	Total (37 CFR 1.16(i))	*	Minus	**	=	X $ =		OR	X $ =	
	Independent (37 CFR 1.16(h))	*	Minus	***	=	X $ =		OR	X $ =	
	☐ Application Size Fee (37 CFR 1.16(s))									
	☐ FIRST PRESENTATION OF MULTIPLE DEPENDENT CLAIM (37 CFR 1.16(j))							OR		
						TOTAL ADD'L FEE		OR	TOTAL ADD'L FEE	

* If the entry in column 1 is less than the entry in column 2, write "0" in column 3.

** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 20, enter "20".

*** If the "Highest Number Previously Paid For" IN THIS SPACE is less than 3, enter "3".

The "Highest Number Previously Paid For" (Total or Independent) is the highest number found in the appropriate box in column 1.

Legal Instrument Examiner:
/LISA THOMAS/

This collection of information is required by 37 CFR 1.16. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, VA 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. **SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.**

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Samsung Bioepis Exhibit No. 1002
Page 8867