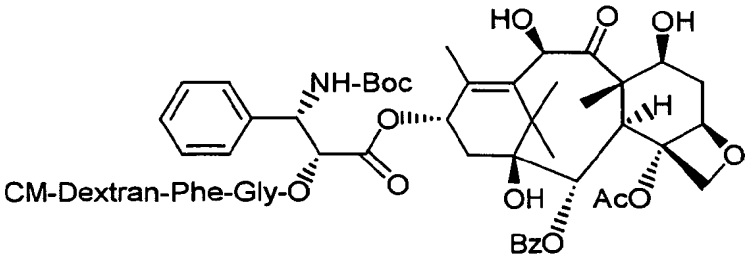
emulsified in purified water (64 ml), and thereto was added 6N aqueous hydrochloric acid solution (32 ml), and the mixture was reacted at room temperature under stirring for 2 hours. The solvent was concentrated, and thereto was added n-propanol to precipitate powdery product. The resulting powdery product was collected by filtration, and recrystallized from aqueous n-propanol to give 7-ethyl-10-[3'-(glycyl-glycyl-glycylamino)propyloxy]-(20S)-camptothecin hydrochloride (2.56 g) as yellow crystals.

(3) CM-Dextran sodium salt (CM-degree = 0.44, 50 g) was dissolved in water (2.5 liters), and the pH value thereof was adjusted to pH 5.0 with 0.2N aqueous hydrochloric acid solution under stirring at 15°C, and thereto was added 7-ethyl-10-[3'-(glycyl-glycyl-glycylamino)propyloxy]-(20S)-camptothecin hydrochloride (4.01 g). To the mixture was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (50 g), during which the pH value of the reaction solution was kept at 5.0-5.5 with 0.2N hydrochloric acid. The mixture was reacted at 15°C under stirred for one hour, and diluted to the total volume of 10 liters with purified water. While the pH value was kept at over pH 4.0, the low molecule fractions were removed by using an ultrafiltration module (ACP-1010, manufactured by Asahi Kasei Industries, Ltd.), and the pH value thereof was adjusted to pH 8 with 0.1N aqueous sodium hydroxide

solution, and then subjected to ion-exchange resin MSC-1 (Na-type, manufactured by Dowex). The fractions containing the desired compounds are concentrated, and filtered through a filter (0.45 μm). The resultant was mixed with ethanol (10 liters) with stirring, and thereto was added dropwise 3M brine (40 ml) under stirring. The resulting precipitates were collected by filtration, and dissolved in purified water (21 liters). The pH value of the solution was adjusted to pH 4.0 with 0.2N aqueous hydrochloric acid solution, and subjected again to ultrafiltration during which the pH value was kept at pH 4.0. The solvent was concentrated to the total volume of 1.5 liter, and filtered through a filter (0.45 μm). The resultant was mixed with ethanol (9 liters), and thereto was added dropwise 3M brine (35 ml) under stirring. The resulting precipitates were collected by filtration, and washed successively with ethanol and acetone, concentrated under reduced pressure to give the desired compound (54.9 g) as pale yellow powder. The content as 10-(3'-aminopropyloxy)-7-ethyl-(20S)-camptothecin hydrochloride was confirmed as 4.2 % by absorption at 367.5 nm. According to the analysis by GPC (Gel Permeation Chromatograph), the average molecular weight of the desired product was 121 kDa, and the degree of distribution (Mw/Mn) was 1.47.

Preparation 2

Preparation of CM-dextran-13-[(2'R,3'S)-3'-N-tert-butoxycarbonyl-3'-phenyl-2'-O-L-phenylalanyl-glycyl-isoserinyl]-10-deacyl-baccatin III:



(Bz is benzoyl group, hereinafter, the same)

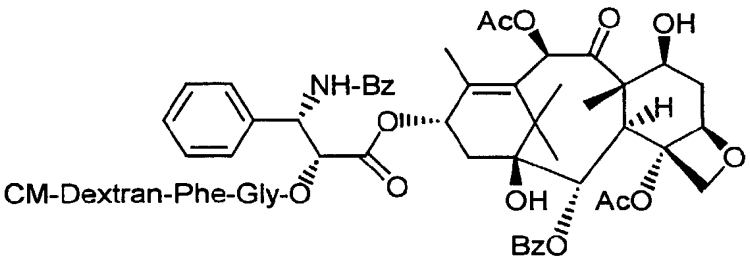
CM-Dextran(2008 mg, CM-degree: 0.47, the average molecular weight: 170 kDa) was dissolved with stirring in purified water (90 ml), and thereto were added 13-[(2'R,3'S)-3'-N-tert-butoxycarbonyl-3'-phenyl-2'-O-L-phenylalanyl-glycyl-isoserinyl]-10-deacyl-baccatin III mesylate (119 mg) and dimethylformamide (90 ml), and the mixture was stirred so as to dissolve. To the mixture was added with stirring 2-ethoxy-1(2H)-quinolinecarboxylic acid (4.0 g), and the mixture was stirred at room temperature overnight. To the reaction solution was added ethanol (720 ml) with stirring, and thereto was further added dropwise 3M brine (1.8 ml) under stirring. The precipitates were collected by centrifugation, and dissolved in water (200 ml), and the pH value of the solution was adjusted to pH 7 with 0.2N aqueous sodium hydroxide solution. The solution was poured into ethanol (800 ml) with stirring, and thereto

was added dropwise 3M brine (4 ml) with stirring. The resulting precipitates were collected by centrifugation, and purified in the same manner as in Preparation 1-(3) to give the desired compound (600 mg) as white powder.

The content of the active substance: 2.4 %(UV method, (λ = 276 nm))

Preparation 3

Preparation of CM-dextran-2'-O-phenylalanyl-glycyl-taxol:



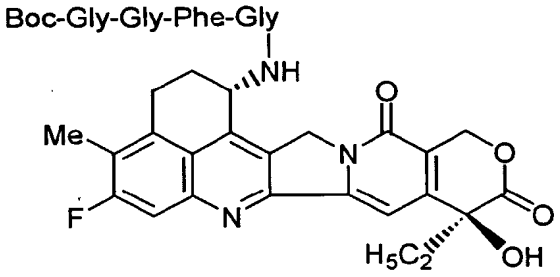
CM-Dextran (1.294g, CM-degree: 0.47, the average molecular weight: 170 kDa) was dissolved with stirring in purified water (70 ml), and thereto were added 2'-O-phenylalanyl-glycyltaxol mesylate (77 mg) and dimethylformamide (70 ml), and the mixture was further stirred so as to dissolve. 2-Ethoxy-1(2H)-quinolinecarboxylic acid (2.59 g) was added to the mixture under stirring, and the mixture was reacted with stirring overnight. The reaction solution was added to ethanol (700 ml) under stirring, and thereto was added dropwise 3M brine (1.4 ml) under stirring. The precipitates were collected by centrifugation, and dissolved in water (240 ml), and mixed with ethanol (1200

ml) under stirring. 3M Brine (4.8 ml) was added drowpise to the mixture under stirring for precipitation. In the same manner, the precipitation was further repeated three times to give the desired product (746 mg) as white powder. The content of the active substance: 4.8 % (UV method (λ = 273 nm))

Preparation 4

Preparation of carboxymethyldextran-polyalcohol-(1S,9S)-1-(glycyl-glycyl-L-phenylalanyl-glycylamino)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]-pyrano[3',4':6,7]indoridino[1,2-b]quinoline-10,13(9H,15H)-dione:

(1) Preparation of (1S,9S)-1-(t-butoxycarbonyl-glycyl-glycyl-L-phenylalanyl-glycylamino)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano-[3',4':6,7]indolidino[1,2-b]quinoline-10,13(9H,15H)-dione:



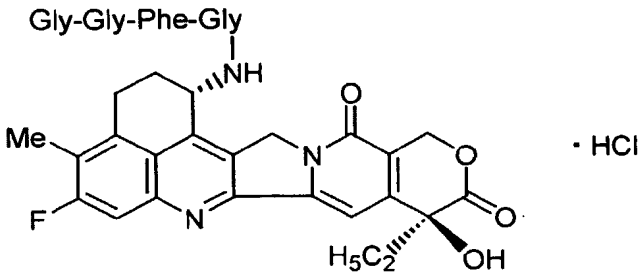
To a solution of (1S,9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano-[3',4':6,7]indolidino[1,2-b]quinoline-10,13(9H,15H)-dione hydrochloride (167 mg; 0.354 mmol), t-butoxycarbonyl-

glycyl-glycyl-L-phenylalanyl-glycine (463 mg; 1.06 mmol) and 1-hydroxybenzotriazole monohydrate (HOBT) (143 mg; 1.06 mmol) in dimethylformamide (DMF) (10ml) were added 1-(3-dimethylaminopropyl)-3-ethylcarbodimimide (EDC) hydrochloride (270 mg; 1.42 mmol), triethylamine (148 µl; 1.06 mmol) and 4-dimethylaminopyridine (DMAP) (5 mg; 0.04 mmol). The reaction mixture was stirred at room temperature for 15 hours, and the solvent was concentrated under reduced pressure. The residue was dissolved in chloroform, and the mixture was washed, dried, and the solvent was evaporated under reduced pressure. The residue was purified by silica gel column chromatography (solvent; chloroform : methanol = 50:1 to 10:1) to give the title compound (228 mg, yield: 75 %) as pale yellow solid.

IR (Nujol); 3290, 1710, 1655 cm^{-1}

ESI-MS; 854 (M+H)

(2) Preparation of (1S,9S)-1-(glycyl-glycyl-L-phenyl-alanyl-glycylamino)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolidino[1,2-b]quinoline-10,13(9H,15H)-dione:



To a solution of (1S,9S)-1-(t-butoxycarbonyl-glycyl-glycyl-L-phenylalanyl-glycylamino)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano-[3',4':6,7]indolidino[1,2-b]quinoline-10,13(9H,15H)-dione (220 mg; 0.258 mmol) in dioxane (4 ml) was added 4N hydrogen chloride solution in dioxane (6 ml) under stirring in an ice bath. The mixture was stirred at room temperature for 16 hours. Diethyl ether (30 ml) was added to the reaction mixture, and the mixture was stirred at room temperature for one hour. The precipitates were collected by filtration, and dried to give the title compound (176 mg, yield; 86 %) as yellow powder.

IR (Nujol); 3250, 1745, 1660, 1605, 1535 cm^{-1}

ESI-MS; 754 (M+H)

(3) Preparation of dextran polyalcohol (PA-Dextran):

Acetic buffer (0.1 M, pH 5.5, 1000 ml) was put into a three-neck round bottom flask (capacity; 3 liters). Dextran T-500® (10.0 g, manufactured by Amersham Pharmacia Biotech AB) was added in small portions to the buffer over a period of 30 minutes at room temperature. The mixture was stirred for about 30 minutes until the solution became clear, and then, the mixture was cooled at 5°C (inner temperature) in a bath.

Separately, to a flask (capacity; 1 liter) were added sodium periodate (33.0 g) and water (1000 ml), and the

mixture was stirred at room temperature, and then cooled at 5°C.

To the above dextran solution was added with stirring the above sodium periodate solution at 5°C, and the mixture was ketp at 5°C for 5 days in a dark place. The excess sodium periodate was removed by adding ethylene glycol (10 ml), and the mixture was further stirred at 5°C for 2 hours. The reaction mixture was cooled to 3°C, and thereto was added 8M aqueous sodium hydroxide solution during which the reaction temperature was kept below 6°C (the pH value of the reaction mixture became over pH 9). To the reaction mixture was added sodium borohydride (14 g) in small portions with stirring, and the mixture was stirred at 5°C overnight. In order to remove the excess sodium borohydride, the pH value of the reaction mixture was adjusted to below pH 5.5 by adding acetic acid thereto at 3 to 6°C, and the mixture was further stirred for 2 hours. The pH value of the reaction mixture was adjusted to about pH 7.8 with 8M aqueous sodium hydroxide solution. The mixture was subjected to dialysis against water (Spectora®/Por 3 membrane, Molecule weight cutoff <3500), and lyophilized to give dextran polyalcohol (8.34 g) as amorphous powder.

(4) Preparation of carboxymethyldextran polyalcohol (CM-PA-Dextran):

Water (155 ml) was put into a three-neck round bottom flask (capacity; 500 ml), and thereto was added with stirring dextran polyalcohol (5.18 g) at room temperature over a period of 10 minutes. The mixture was stirred for about 10 to 30 minutes until the mixture became clear, and then sodium hydroxide (pellet, 97.0 %, 21.8 g) was added to the dextran polyalcohol solution in small portions under stirring, during which the inner temperature was kept at 30 to 40°C in an ice bath. The reaction flask was put in a bath, and the mixture was stirred at 30°C. Chloroacetic acid (31.1 g) was added with stirring in small portions into the reaction mixture at 30 to 40°C. After the addition, the mixture was further stirred at 30°C in a bath for 20 hours. The reaction mixture was cooled in an ice bath, and the mixture was neutralized by adding thereto acetic acid under stirring (i.e., the pH value was adjusted to below pH 9).

Water (160 ml) was added to the mixture, and the mixture was subjected to dialysis against water (Spectora®/Por 3 membrane, Molecule weight cutoff <3500), and lyophilized to give carboxymethydextran polyalcohol (6.53 g) as amorphous powder.

(5) Preparation of carboxymethyldextran-polyalcohol-(1S,9S)-1-(glycyl-glycyl-L-phenylalanyl-glycylamino)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-

benzo[de]pyrano[3',4':6,7]indolidino[1,2-b]quinoline-10,13(9H,15H)-dione:

Water(40 ml) was put into a round bottom flask (capacity; 100 ml), and thereto was added carboxymethyldextran polyalcohol (1.0g) at room temperature with stirring over a period of 5 minutes. The mixture was stirred about 30 minutes until the mixture became clear. A solution of (1S,9S)-1-(glycyl-glycyl-L-phenylalanyl-glycylamino)-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]pyrano[3',4':6,7]indolidino[1,2-b]quinoline-10,13(9H,15H)-dione in dimethylformamide (100 mg/10 ml) was added with stirring to the mixture, and further added thereto dimethylformamide (15 ml), and the mixture was stirred for 10 minutes. To the mixture was added dropwise with stirring a solution of 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ) in dimethyl-formamide (1.0 g/10 ml) at room temperature, and the mixture was further stirred for 18 hours. The reaction mixture was subjected to dialysis against water (Spectora®/Por 3 membrane, Molecule weight cutoff <3500), and further purified by cation exchange column (BioRad AG® MP-50 column, Na-type, 30 ml). The main fraction was subjected to dialysis (Spectora®/Por 3 membrane, Molecule weight cutoff <3500), and lyophilized to give a crude product, which was pulverized with acetone, collected by

filtration, and dried to give the desired product (904 mg) as pale yellow powder.

INDUSTRIAL APPLICABILITY

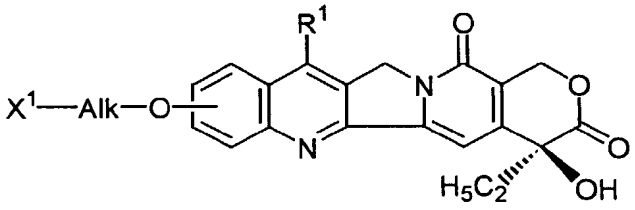
The pharmaceutical composition of the present invention may highly accumulate at the site such as lymph node or the liver to which cancers may spread, and suppressively act on the growth of tumor cells without affecting on the normal cells, and hence, the pharmaceutical composition of the present invention is useful in the inhibition of metastasis, particularly in the inhibition of lymph node metastasis or liver metastasis, or prevention of reoccurrence of a malignant tumor.

In addition, the present pharmaceutical composition may exhibit its effects not only before the onset of metastasis but also after the onset of metastasis. Therefore, the present pharmaceutical composition is also useful in the inhibition of metastasis or prevention of reoccurrence of a malignant tumor after topical therapy (e.g., surgery, radiation therapy, thermotherapy, cryotherapy, laser burning therapy, etc.).

CLAIMS

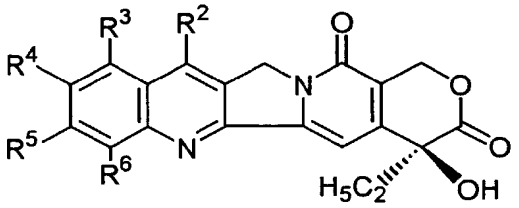
1. A pharmaceutical composition for inhibiting the metastasis or preventing the recurrence of a malignant tumor, which comprises as the active ingredient a polysaccharide derivative comprising a polysaccharide having a carboxyl group bound to an active substance having an anti-tumor activity via an amino acid or a peptide consisting of 2 to 8 amino acids which are the same or different, or a salt thereof.

2. The pharmaceutical composition according to claim 1, wherein the active substance having an anti-tumor activity is a camptothecin derivative of the formula (I):



(I)

wherein R^1 is a substituted or unsubstituted lower alkyl group, X^1 is a group of the formula: $-NHR^2$ (R^2 is a hydrogen atom or a lower alkyl group) and Alk is a straight chain or branched chain C_{1-6} alkylene group having optionally an oxygen atom in the chain thereof, or a compound of the formula (II):



(II)

wherein two groups of R^2 to R^6 being adjacent each other combine to form a lower alkylene group, and one of the carbon atoms of said lower alkylene group is substituted by an amino group, and the remaining three groups of R^2 to R^6 are a hydrogen atom, a lower alkyl group or a halogen atom.

3. The pharmaceutical composition according to claim 1 or claim 2, wherein the polysaccharide having a carboxyl group is a carboxy-C_{1-4} alkyldextran or a carboxy-C_{1-4} alkyldextran polyalcohol.

4. The pharmaceutical composition according to claim 1 or claim 2, wherein the polysaccharide having a carboxyl group is a carboxy-C_{1-4} alkyldextran.

5. The pharmaceutical composition according to any one of claims 1, 2, 3 and 4, wherein the peptide is a member selected from the group consisting of glycyl-glycyl-L- or D-phenylalanyl-glycine, glycyl-glycine, glycyl-glycyl-glycine, glycyl-glycyl-glycyl-glycine, glycyl-glycyl-glycyl-glycyl-glycine, L- or D-phenylalanyl-glycine, L- or D-tyrosyl-glycine, L- or D-leucyl-glycine, L- or D-phenylalanyl-citrulline and L- or D-valyl-citrulline.

6. The pharmaceutical composition according to claim 1,

wherein the polysaccharide having a carboxyl group is a carboxymethylated dextran, the active substance having an anti-tumor activity is 10-(3'-aminopropyloxy)-7-ethyl-(20S)-camptothecin, and the peptide is glycyl-glycyl-glycine.

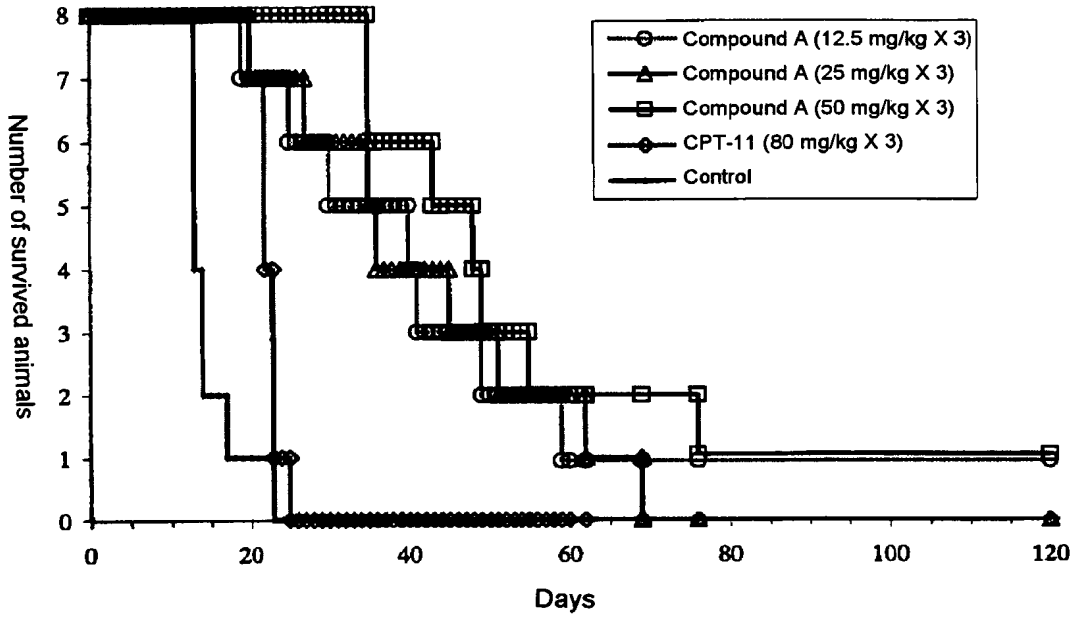
7. The pharmaceutical composition according to claim 1, wherein the polysaccharide having a carboxyl group is a carboxy-C_{1-4} alkyldextran polyalcohol, the active substance having an anti-tumor activity is (1S, 9S)-1-amino-9-ethyl-5-fluoro-2,3-dihydro-9-hydroxy-4-methyl-1H,12H-benzo[de]-pyrano[3',4':6,7]indolidino[1,2-b]quinoline-10,13(9H,15H)-dione, and the peptide is glycyl-glycyl-L- or D-phenyl-alanyl-glycine.

8. The pharmaceutical composition according to any one of claims 1 to 7, which is a pharmaceutical composition for inhibiting the metastasis of a malignant tumor.

9. The pharmaceutical composition according to any one of claims 1 to 7, which is a pharmaceutical composition for preventing the recurrence of a malignant tumor.

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FIG 1


Compound A (12.5 mg/kg X 3)
Compound A (25 mg/kg X 3)
Compound A (50 mg/kg X 3)
CPT-11 (80 mg/kg X 3)
Control
Number of survived animals
0
1
2
3
4
5
6
7
8
0
20
40
60
80
100
120
Days

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/JP 02/08309

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K47/48 A61P35/04

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 7 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)
EPO-Internal, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 757 049 A (TANABE SEIYAKU CO) 5 February 1997 (1997-02-05) claims 1,4-6,9,33,36; examples 22-30,45,47,51-69,81-92,114-158 ---	1-6,8,9
X	EP 0 781 781 A (TANABE SEIYAKU CO) 2 July 1997 (1997-07-02) page 8, lines 30-32; examples (see in particular examples 7,77-93); claims 1,4,6-12,15 ---	1-5,7-9
X	EP 1 080 732 A (DAIICHI SEIYAKU CO) 7 March 2001 (2001-03-07) page 8, lines 44-45; page 9, line 1 -page 10, line 45; claims 1-3,6-13; examples 1,5-7 --- -/--	1-3,5, 7-9

[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

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"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

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"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
23 October 2002	06/11/2002

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	Fausti, S

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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 916 348 A (DRUG DELIVERY SYSTEM INST LTD ;DAIICHI SEIYAKU CO (JP)) 19 May 1999 (1999-05-19) page 9, line 1 -page 10, line 20; claims 1-10,14-18; examples 1,8-10,15,17,23,27-29,34-41,44,46,47,52-55 ---	1-3,5,8, 9
X	EP 0 640 622 A (DRUG DELIVERY SYSTEM INST LTD) 1 March 1995 (1995-03-01) claims 1,4,6,8,14,15; examples 9,13,14 ---	1,3-5,8, 9
X	SCHECHTER B ET AL: "LIVER ACCUMULATION OF TNP-MODIFIED STREPTAVIDIN AND AVIDIN: POTENTIAL USE FOR TARGETED RADIO- AND CHEMOTHERAPY" JOURNAL OF DRUG TARGETING, HARWOOD ACADEMIC PUBLISHERS GMBH, DE, vol. 4, no. 3, 1996, pages 171-179, XP000783534 ISSN: 1061-186X abstract; page 173, left-hand column, 3rd paragraph; page 176, right-hand column, lines 6-9,20-24 -----	1,3,4,8, 9

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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International Application No
PCT/JP 02/08309

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/JP 02/08309

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		PT 640622 T	30-11-2000

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 May 2003 (30.05.2003)

PCT

(10) International Publication Number
WO 03/043583 A2

(51) International Patent Classification[7]: A61K

(21) International Application Number: PCT/US02/37223

(22) International Filing Date:
20 November 2002 (20.11.2002)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/331,750 20 November 2001 (20.11.2001) US

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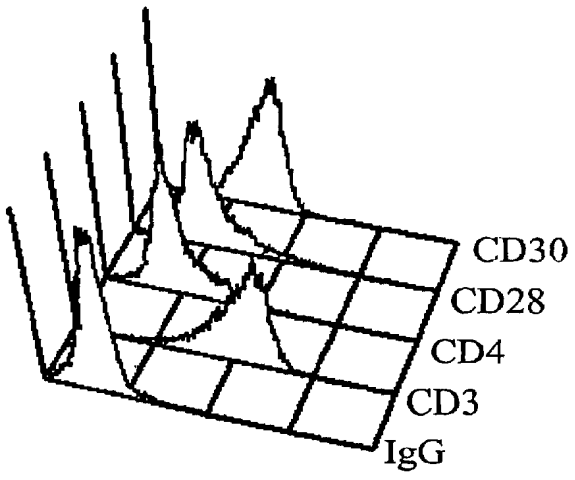
(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZM, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (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*

[Continued on next page]

(54) Title: TREATMENT OF IMMUNOLOGICAL DISORDERS USING ANTI-CD30 ANTIBODIES



	Mean fluorescence intensity
IgG	6
CD3	152
CD4	16
CD28	21
CD30	30

(57) Abstract: The present invention relates to methods for the treatment of immunological disorders other than cancer, comprising administering proteins characterized by their ability to bind to CD30 and exert a cytostatic or cytotoxic effect on an activated lymphocyte. Such proteins include monoclonal antibodies AC10 and HeFi1, AC10 and HeFi-1 derivatives, and antibodies that compete with AC10 and HeFi-1 for binding to CD30. Other such proteins include multivalent anti-CD30 antibodies and anti-CD30 antibodies conjugated to cytotoxic agents. Treatment modalities with the antibodies of the invention are also provided.

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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.

TREATMENT OF IMMUNOLOGICAL DISORDERS USING ANTI-CD30 ANTIBODIES

This application claims benefit under 35 U.S.C. § 119(e) of U.S. provisional application no. 60/331,750, filed November 20, 2001, which is incorporated by reference herein in its entirety.

1. FIELD OF THE INVENTION

This invention relates to methods and compositions for inducing cell death or stasis in activated lymphocytes using CD30-binding proteins, and applications for these methods and compositions for the treatment of immunological diseases such as autoimmunity, allergy, chronic inflammatory reactions, and graft versus host disease (GVHD). The present invention further relates to methods and compositions for treatment of immunological disorders by eliminating CD30-expressing lymphocytes using conjugates of CD30-binding proteins and cytotoxic agents. Exemplary CD30-binding proteins that are useful in the methods and compositions of the present invention include the anti-CD30 antibodies AC10 and HeFi-1 and conjugates of AC10 or HeFi-1 and cytotoxic agents.

2. BACKGROUND OF THE INVENTION

2.1 LYMPHOCYTES

Subsequent to antigenic stimulation and cellular expansion, naïve T cells can develop into phenotypically distinct effector cells. Effector T cells (helper or cytotoxic T cells) that secrete pro-inflammatory cytokines like IFNγ and lymphotoxin are collectively designated as Th_1 or Tc_1 cells, and effector T cells that secrete IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13 are known as Th_2 or Tc_2. Th_1/Tc_1 cytokines induce cell-mediated responses including the activation of CTL and monocyte/macrophages, whereas Th_2/Tc_2 cytokines induce humoral immune responses by enhancing antibody production by B cells. As a result, uncontrolled Th_1/Tc_1 responses are usually manifested as organ-specific autoimmune responses such as rheumatoid arthritis and diabetes. On the other

hand, uncontrolled Th_2/Tc_2 responses are usually associated with allergic reactions and systemic autoimmune diseases like systemic lupus erythematosus. The two polarized T cell subsets can modulate each others activity in a reciprocal fashion - the presence of Th_2/Tc_2 cytokines can partially alleviate symptoms resulted from cell-mediated autoimmunity and vice versa (Seder and Mosmann, 1999, in 'Fundamental Immunolgy', 4th Ed., pp 879-908; O'Gara and Arai, 2000, Trends Cell Biol. 10:542-550).

2.2 CD30

The leukocyte activation marker CD30 is a 105–120 kDa integral membrane glycoprotein and a member of the tumor necrosis factor receptor (TNF-R) superfamily. This family of key immunoregulatory molecules includes CD27, CD40, CD95, OX40, TNF-R1 and TNF-R2. Originally identified on Reed-Sternberg cells in Hodgkin's disease (HD) using the Ki-1 monoclonal antibody (mAb) (Stein *et al.*, 1985, Blood, 66, 848-858; Laudewitz *et al.*, 1986, J. Invest. Dermatol. 86:350-354), CD30 has subsequently been found on anaplastic large cell lymphoma (ALCL), subsets of non-Hodgkin's lymphomas (NHL), as well as in rare solid tumors such as embryonal carcinomas and seminomas (Chiarle *et al.*, 1999, Clin. Immunol. 90:157-164). Under normal conditions expression of CD30 is restricted to activated T and B cells and absent from resting lymphocytes, resting monocytes and from normal cells outside of the immune system. CD30 is expressed at high levels by activated cells in autoimmune disease, and shed CD30 from these cells is detectable in the circulation of patients suffering from diseases including rheumatoid arthritis (Gerli *et al.*, 2000, J. Immunol. 164, 4399-4407), multiple sclerosis (McMillan*et al.*, 2000, Acta Neurol. Scand. 101:239-243) and systemic sclerosis (Ihn *et al.*, J. Rheumatol. 27:698-702). Histological examination for CD30 expression identifies rare, large lymphoid cells in sections of lymph node, tonsil, thymus, and decidual endometrial cells at the placental interface (Durkop *et al.*, 2000, J. Pathol. 190:613-618). CD30 is transiently expressed on T cells in culture after mitogen activation or antigen receptor crosslinking (Horie and Watanabe, 1998, Sem. Immunol. 10:457-470), and is constitutively expressed following some viral infections, *e.g.*, HIV (Romagnani *et al.*, 1996, Immunol. Lett. 51:83-88).

A ligand for CD30 (CD30L) has been identified. CD30L is a type II transmembrane protein, and it shares sequence and structural homologies with members of the TNF superfamily (Smith *et al.*, 1993, Cell, 73, 1349-1360). It is believed that CD30L, like TNFα, also exists as a trimer, and CD30L-CD30 interaction induces trimerization of CD30 to initiate signal transduction to $CD30^+$ cells. Expression of CD30L has been demonstrated in activated T cells, neutrophils, eosinophils, resting B cells, epithelial cells and Hassal's corpuscles of the thymic medulla, and some leukemic cells (Younes *et al.*, Br. J. Haematol., 93:569-571; Pinto *et al.*, 1996, Blood, 88:3299-3305; Grüss *et al.*, 1996, Am. J. Pathol. 149, 469-481; Gattei *et al.*, 1997, Blood, 89, 2048-2059; Romagnani *et al.*, 1998, Blood 91:3323-3332; Wiley *et al.*, 1996, J. Immunol., 157:3635-3639).

The lymphocyte surface antigen CD30 is an activation marker transiently displayed on activated B and T cells and constitutively expressed on some malignant hematologic cells and on chronically activated -cells in several autoimmune diseases. Its role in lymphocyte regulation is believed to be one of attenuation, as lack of CD30 in knock-out animals results in hyperresponsiveness to immune stimuli, whereas over expression of the transgene in the thymus results in increased thymocyte depletion. Thus, elimination or attenuation of activated lymphocytes via CD30-targeted therapy could be efficacious in controlling autoimmune and chronic inflammatory diseases.

The complexity of CD30 in regulating lymphocyte survival has lead to disparate accounts of its effects in model systems. Duckett and Thompson (1997, Genes Dev. 11:2810-21) have shown the initial effect of CD30 signaling is to promote survival by recruitment of TRAF1 and TRAF2. This signal transduction complex promotes growth and survival-gene expression *via* NF-κB. Following this activation, TRAFs complexed with CD30 are then degraded, compromising the ability of the cells to further activate NF-κB. Consequently, the cells become sensitized to apoptosis induced by ligation of TNF-R1 (Duckett and Thompson, 1997, Genes Dev. 11:2810-21; Arch *et al.*, 2000, Biochem. Biophys. Res. Commun. 272:936-945). Sensitization is dependent on a TRAF2 binding site within the cytoplasmic domain of CD30 and cellular degradation of TRAF2 is coincident with the onset of apoptosis.

2.3 ROLE OF CD30 IN THE IMMUNE SYSTEM

2.3.1 NORMAL IMMUNE SYSTEM

The *in vivo* function of CD30 is not clearly understood. Ligation of CD30 *in vitro* has been shown to induce either cellular proliferation or growth inhibition (Gruss *et al.*, 1994, Blood, 83, 2045-2056). Ligation of CD30 on T cells has been demonstrated to regulate a variety of *in vitro* T cell functions. Anti-CD30 mAbs co-stimulate with T cell antigen receptor ligation to augment cellular proliferation in T cells that have been primed by either antigens (Del Prete *et al.*, 1995, J. Exp. Med., 182:1655-1661) or anti-CD3 antibodies (Gilfillan *et al.*, 1998, J. Immunol. 160:2180-2187). CD30 signaling also promotes cytokine production in T cells. Expression of interleukin (IL)-2, IL-4, IL-5, IFNγ, and TNFα are enhanced by CD30 signals in PHA activated peripheral T cells (Grüss and Hermann, 1996, Leukemia Lymphoma, 20:397-409), T helper (Th) clones (Del Prete *et al.*, 1995, J. Exp. Med., 182:1655-1661; Bengtsson *et al.*, 2000, Scand. J. Immunol. 52, 595-601), cytotoxic T cell (Tc/CTL) lines (Bowen *et al.*, 1996, J. Immunol. 156:442-449), and γδ T cells (Biswas *et al.*, 2000, Eur. J. Immunol. 30:2172-2180). CD30 may also play an important role in stimulating HIV replication. Cross-linking of CD30 by mAbs induces NF-κB activation and HIV production by a T cell line chronically infected with HIV (Biswas *et al.*, 1995, Immunity, 2, 587-596). $CD4^+$, HIV-infected T cells derived from patients also respond to anti-CD30 mAbs or CD30L stimulation to produce HIV (Maggi *et al.*, 1995, Immunity 3:251-255).

CD30 has been implicated in the activation-induced cell death of T cells. Ligation of a CD8-CD30 chimera expressed in a T cell hybridoma enhanced apoptosis mediated by the T cell antigen receptor (Lee *et al.*, 1996, J. Exp. Med. 183:669-674). In a second model of agonist withdrawal induced apoptosis in murine CD8 T cells, blockage of CD30 signaling using a CD30-Ig fusion protein partially prevented CD8 T cells from undergoing apoptosis (Telford *et al.*, 1997, Cell. Immunol. 182:125-136). Results obtained from animal models also suggest a role of CD30 in the induction of apoptosis in thymocytes, a negative selection process for the deletion of auto-reactive T cells (Amakawa *et al.*, 1996, Cell 84:551-562). In $CD30^{-/-}$ null mice negative selection is severely diminished, giving rise to increased thymocyte numbers (Amakawa *et al.*, 1996 Cell 84:551-562); one demonstrated consequence being that CD30-deficient CD8-

positive T cells are orders of magnitude more autoreactive in their capacity to cause autoimmune diabetes (Kurts *et al.*, 1999, Nature 398:341-344). Conversely, over-expression of CD30 has been shown to promote programmed cell death in thymocytes, and hence enhances negative selection of auto-reactive T cells in the thymus (Chiarle *et al.*, 1999, J. Immunol. 163:194-205), and prevent T cell autoresponses to non-lymphoid tissue in the periphery (Heath *et al.*, 1999, Immunol. Rev. 169:23-29).

2.3.2 THE DISEASE STATE

Because CD30 was demonstrated to be preferentially expressed on human T cell clones secreting Th_2 cytokines (Del Prete *et al.*, 1995, FASEB J., 9, 81-86), and cross-linking of CD30 on T cells promoted the development of Th_2-like T cells (Del Prete *et al.*, 1995, J. Exp. Med., 182, 1655-1661), it has been postulated that immunological disorders involving Th_2 cytokines may result from the dysregulation of $CD30^+$ T cells.

The expression of CD30 has been shown to be increased or altered in a variety of autoimmune and inflammatory diseases including atopic allergy (atopic dermatitis, atopic asthma, rhinoconjunctivitis, allergic rhinitis), systemic lupus erythematosus, systemic sclerosis (scleroderma), graft versus host disease (GVHD), HIV and EBV infection, measles, Omenn's syndrome, ulcerative colitis, rheumatoid arthritis, multiple sclerosis, psoriasis, Hashimoto's thyroiditis, primary biliary cirrhosis, Sjogren's syndrome, Wegener's granulomatosis, and tuberculosis (Gruss *et al.*, 1997, Immunol. Today 18:156-163; Horie and Watababe, 1998, Sem. Immunol. 10:457-470; Bengtsson, 2001, Allergy 56:593-603; Gerli *et al.*, 2001, Trends Immunol. 22:72-77).

CD30+ infiltrating T cells have been found in lesions and inflamed sites of atopic dermatitis (Caproni *et al.*, 1997, Allergy 52:1063-1070; Cavagni *et al.*, 2000, Allergy Immunol. 121:224-228), atopic asthma (Blanco Quirós *et al.*, 1999, Pediatr. Allergy Immunol. 10:235-240), GVHD (D'Elios *et al.*, 1997, J. Lewukoc. Biol. 61:539-544), tuberculosis (Munk *et al.*, 1997, Int. Immunol. 9:713-720), primary biliary cirrhosis (Harada *et al.*, 1999, J. Gastroenterol. Hepatol. 14:1197-1202), measles (Vinante *et al.*, 1999, Haematologica 84:683-689), rheumatoid arthritis (Gerli *et al.*, 2000, J. Immunol. 164:4399-4407; Gerli *et al.*, 1995, Clin. Exp. Immunol. 102:547-550), and psoriasis (Ferenczi *et al.*, 2000, J. Autoimmun. 14:63-78). Although CD30 expression is strictly

restricted to a small percentage of activated lymphocytes in normal situations, increased expression of CD30 mRNA has been reported in atopic asthma and allergic rhinitis (Esnault *et al.*, 1996, Clin. Exp. Immunol. 106:67-72). Correlation between altered expression of CD30 and immune disorders is further underscored by the high plasma levels of soluble CD30 (sCD30) found in many of the aforementioned diseases (Gruss *et al.*, 1997, Immunol. Today 18:156-163; Horie and Watababe, 1998, Sem. Immunol. 10:457-470; Bengtsson, 2001, Allergy 56:593-603; Gerli *et al.*, 2001, Trends Immunol. 22:72-77). In some of these diseases high plasma levels of sCD30 are not accompanied with circulating $CD30^+$ T cells, but expansion of $CD30^+$ T cells in the disease tissue is believed to be a potential source of sCD30 (Mavilia, 1997, Am. J. Pathol. 151:1751-1758).

Several diseases have been demonstrated to involve both Th_2 cytokines and $CD30^+$ T cells (D'Elios *et al.*, 1997, J. Leukoc. Biol. 61:539-544). Most patients with atopic dermatitis show increased production of Th_2 cytokines with parallel increases in the serum IgE levels and augmented numbers of circulating eosinophils (Leung, 1995, J. Allergy Clin. Immunol. 96:302-319). An increased frequency of allergen-specific Th_2 cells producing IL-4, IL-5, and IL-13 can also be detected in the peripheral blood of atopic dermatitis patients (Kimura *et al.*, 1998, J. Allergy Clin. Immunol., 101, 84-89). A correlation between CD30 expression and Th_2 cytokine production has been noted in atopic dermatitis patients which has led to the suggestion that CD30 expression in circulating T cells might serve as an *in vivo* marker for the Th_2-dominated condition (Yamamoto *et al.*, 2000, Allergy, 55, 1011-1018). Allergen induced IL-4 production in atopic asthma correlates with CD30 expression in PBMC isolated from atopic asthma patients (Leonard *et al.*, 1997, Am. J. Respir. Cell Mol. Biol., 17, 368-375). This has also been shown in bronchial alveolar lavage γδ T cells from asthma patients (Spinozzi *et al.*, 1995, Mol. Med. 1:821-826). Likewise, in systemic sclerosis, a disease characterized by elevated serum levels of sCD30 and CD30 expression in skin lesions, IL-4 mRNA can be detected by *in situ* hybridization in lymphocytes infiltrating skin lesions while most $CD4^+$ T cell clones generated from skin infiltrates also demonstrate a Th_2 cytokine profile (Mavalia *et al.*, 1997, Am. J. Path. 15, 1751-1758). In patients infected with HIV, a disproportionately higher frequency of $CD8^+/CD30^+$ cells producing Th_2 cytokines could

be detected in peripheral blood when compared to healthy donors (Manetti *et al.*, 1994, J. Exp. Med. 180:2407-2411). The emergence of a Th_2-type immunity in HIV infected patients also appears to be correlated with the immunopathology associated with disease progression (Rizzardi *et al.*, 1998, Clin. Exp. Immunol. 114:61-65)

In contrast to the Th_2-related diseases, the role of $CD30^+$ in Th_1-related diseases is not as well defined. High levels of sCD30 are found in the CSF of patients with multiple sclerosis and the circulation of rheumatoid arthritis patients (McMillan *et al.*, 2000, Acta Neurol. Scand. 101:239-243; Gerli *et al.*, 2000, J. Immunol. 164:4399-4407). In these diseases, both Th_1 cytokine driven responses, high circulating levels of CD30 are associated with disease remission, suggesting that Th_2 cytokines contributed by $CD30^+$ T cells may counteract the pathogenic activities of Th_1 cytokines (McMillan *et al.*, 2000, Acta Neurol. Scand. 101:239-243; Gerli *et al.*, 2000, J. Immunol. 164:4399-4407). More recent experiments examining CD30 expression on T cell subsets determined that upon cellular activation Th_1 cells also express CD30 (Hamman *et al.*, 1996, J. Immunol. 156:1387-1391; Bengtsson *et al.*, 1995, J. Leukoc. Biol. 58:683-689). An agonistic anti-CD30 mAb can stimulate the production of the Th_1 cytokine IFNγ in $CD30^+$ Th_1 cells (Bengtsson *et al.*, 2000, Scad. J. Immunol. 52:595-601). In atopic dermatitis, evidence is available to demonstrate a shift from the predominance of Th_2 cytokine production by T cell infiltrating skin lesions to the co-expression of Th_2 and Th_1 cytokines during disease progression (Grewe *et al.*, 1998, Immunol. Today 19:359). Hence CD30 T cells may also play a role in the production of Th_1 cytokines in Th_1-related diseases.

2.4 IMMUNOTHERAPEUTICS

Sustained activation of lymphocytes against self-antigens or allergens and the failure to terminate ongoing immune responses subsequent to the clearance of antigens are the underlying causes of the pathologies seen in autoimmune diseases, allergic reactions, and chronic inflammatory reactions. A variety of therapeutic regimens including antimetabolites, steroids, and anti-inflammatory agents are available for the treatment of autoimmune, allergic, and inflammatory diseases. Although these drugs are efficacious in alleviating symptoms, none of them work by specifically eliminating the pathogenic cells and most of them have severe side effects on patients.

Elimination or attenuation of activated lymphocytes bearing CD30 could be efficacious in controlling autoimmune and chronic inflammatory diseases. However, despite the above evidence for a role of CD30 in immune disorders, targeting CD30 by mAb has not been demonstrated to be effective in treating such disorders. Agents that are capable of eliminating or attenuating CD30-bearing activated lymphocytes would be highly desirable in the treatment of immunological disorders.

3. SUMMARY OF THE INVENTION

The present invention is based on the surprising discovery of a novel activity associated with certain classes of anti-CD30 antibodies. The novel activity is the ability of anti-CD30 antibodies to kill or inhibit the growth of activated lymphocytes, in certain instances by signaling through the CD30 receptor pathway. In certain preferred embodiments, the antibodies of the invention are able to induce apoptosis or growth arrest of activated lymphocytes as monospecific antibodies, in the absence of conjugation to cytotoxic reagents, and/or in the absence of cells other than the CD30-expressing lymphocytes (*e.g.*, in the absence of effector cells).

Accordingly, the present invention provides methods for the treatment of an immunological disorder in a subject, preferably wherein the immune disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) a first antibody that (i) immunospecifically binds CD30 and (ii) induces CD30 signaling in a lymphocyte and/or exerts a cytostatic or cytotoxic effect on an activated lymphocyte; and (b) a pharmaceutically acceptable carrier. The antibody can be human, humanized or chimeric. In one embodiment, the antibody is multivalent. In a preferred embodiment, the antibody competes for binding to CD30 with monoclonal antibodies AC10 or HeFi-1.

The present invention further provides methods for the treatment of an immunological disorder in a subject, preferably wherein the immune disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) a first antibody that (i) immunospecifically binds CD30 and (ii) induces CD30 signaling in a lymphocyte and/or exerts a cytostatic or cytotoxic effect on an activated lymphocyte; and (b) a pharmaceutically acceptable

carrier; said methods further comprising administering an agent that enhances or potentiates the cytostatic or cytotoxic effect of the first antibody. In certain embodiments, the agent that potentiates the cytostatic or cytotoxic effect of the first antibody is a second antibody, a ligand that binds to a receptor or receptor complex expressed on activated lymphocytes, or an immunosuppressive agent. Examples of such reagents and methods of their use are further described *infra*.

The present invention thus provides methods for the treatment of an immunological disorder in a subject, preferably wherein the immune disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) a first antibody that (i) immunospecifically binds CD30 and (ii) induces CD30 signaling in a lymphocyte and/or exerts a cytostatic or cytotoxic effect on an activated lymphocyte; and (b) a pharmaceutically acceptable carrier; said methods further comprising administering a second antibody to the subject. In a preferred embodiment, the second antibody recognizes a non-CD30 receptor or receptor complex expressed on activated lymphocytes.

In certain specific embodiments, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1; and (b) a pharmaceutically acceptable carrier.

In other specific embodiments, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises SEQ ID NO:2 (the heavy chain variable region of the anti-CD30 antibody AC10); and (b) a pharmaceutically acceptable carrier.

In other specific embodiments, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for

said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises one, two or all of: SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8 (the heavy chain CDRs of the anti-CD30 antibody AC10), or variants of SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8 that differ from SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8 by one, two or three amino acids; and (b) a pharmaceutically acceptable carrier.

In other specific embodiments, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises SEQ ID NO:18 (the heavy chain variable region of the anti-CD30 antibody HeFi-1); and (b) a pharmaceutically acceptable carrier.

In other specific embodiments, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises one, two or all of: SEQ ID NO:20, SEQ ID NO:22 and SEQ ID NO:24 (the heavy CDRs of the anti-CD30 antibody HeFi-1), or variants of SEQ ID NO:20, SEQ ID NO:22 and SEQ ID NO:24 that differ from SEQ ID NO:20, SEQ ID NO:22 and SEQ ID NO:24 by one, two or three amino acids; and (b) a pharmaceutically acceptable carrier.

In yet other specific embodiments, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, wherein said antibody is conjugated to a cytotoxic agent; and (b) a pharmaceutically acceptable carrier.

In certain specific embodiment of the present invention, the anti-CD30 antibody is an agonistic antibody. In another specific embodiment of the present

invention, the anti-CD30 antibody is not a non-agonistic antibody. In another specific embodiment, the anti-CD30 antibody does not block binding of CD30 ligand to CD30.

In a preferred embodiment, where a second antibody that recognizes a non-CD30 receptor or receptor complex is administered to the subject, such an antibody is capable of enhancing the cytotoxic or cytostatic effect of the CD30 antibody. While not bound by any theory, such a second antibody enhances the cytotoxic or cytostatic effect of the CD30 antibody by delivering a cytostatic or cytotoxic signal to the activated lymphocytes. Exemplary receptors or receptor complexes include an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein. Non-limiting examples of suitable immunoglobulin superfamily members are CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, and ICOS. Non-limiting examples of suitable TNF receptor superfamily members are CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and APO-3. Non-limiting examples of suitable integrins are CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, and CD104. Non-limiting examples of suitable lectins are C-type, S-type, and I-type lectin.

In certain preferred embodiments, antibodies useful in the present methods, *i.e.*, antibodies that bind to CD30 and exert a cytostatic or cytotoxic effect on an activated lymphocyte, are bispecific antibodies. In a specific embodiment, the bispecific antibodies bind to both CD30 and a non-CD30 receptor or receptor complex expressed on activated lymphocytes. Preferably, the portion of the bispecific antibody that binds to the non-CD30 receptor or receptor complex is capable of enhancing the cytotoxic or cytostatic effect of the CD30 antibody. The non-CD30 binding portion of the bispecific antibody preferably enhances the cytotoxic or cytostatic effect of the CD30 antibody by delivering a cytostatic or cytotoxic signal to the activated lymphocytes. The receptor or receptor complex can comprise an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein. Non-limiting

examples of suitable immunoglobulin superfamily members are CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, and ICOS. Non-limiting examples of suitable TNF receptor superfamily members are CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and APO-3. Non-limiting examples of suitable integrins are CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, and CD104. Non-limiting examples of suitable lectins are C-type, S-type, and I-type lectin.

In other embodiments, the therapeutic methods of the present invention further comprise administering to the subject a ligand that binds to a receptor or receptor complex expressed on activated lymphocytes, concurrently or successively with the anti-CD30 antibody. Preferably, the ligand is capable of enhancing the cytotoxic or cytostatic effect of the CD30 antibody, for example by delivering a cytostatic or cytotoxic signal to the activated lymphocytes. The receptor or receptor complex can comprise an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein. Non-limiting examples of suitable immunoglobulin superfamily members are CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, and ICOS. Non-limiting examples of suitable TNF receptor superfamily members are CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and APO-3. Non-limiting examples of suitable integrins are CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, and CD104. Non-limiting examples of suitable lectins are C-type, S-type, and I-type lectin. Ligands that bind to the foregoing receptors are known to those of skill in the art.

In certain specific embodiments of the present therapeutic methods, the anti-CD30 antibody is a fusion protein comprising the amino acid sequence of a second protein that is not an antibody. In a preferred embodiment, the second protein confers multivalent binding properties to the CD30 antibody.

In other embodiments, the therapeutic methods of the present invention further comprise administering to the subject a cytostatic, cytotoxic, and/or immunosuppressive agent. In one embodiment, the immunosuppressive agent is gancyclovir, acyclovir, etanercept, rapamycin, cyclospórine or tacrolimus. In other embodiments, the immunosuppressive agent is an antimetabolite, a purine antagonist (*e.g.*, azathioprine or mycophenolate mofetil), a dihydrofolate reductase inhibitor (*e.g.*, methotrexate), a glucocorticoid. (*e.g.*, cortisol or aldosterone), or a glucocorticoid analogue (*e.g.*, prednisone or dexamethasone). In yet other embodiments, the immunosuppressive agent is an alkylating agent (*e.g.*, cyclophosphamide). In yet other embodiments, the immunosuppressive agent is an anti-inflammatory agent, including but not limited to a cyclooxygenase inhibitor, a 5-lipoxygenase inhibitor, and a leukotriene receptor antagonist.

In certain preferred embodiments, antibodies useful in the present methods, *i.e.*, antibodies that bind to CD30 and induce CD30 signaling in a lymphocyte and/or exert a cytostatic or cytotoxic effect on an activated lymphocyte, are conjugated to a cytostatic, cytotoxic or immunosuppressive agent. Such conjugated antibodies are sometimes referred to herein as anti-CD30 antibody-drug conjugates ("ADC" or "ADCs") or anti-CD30 antibody-cytotoxic agent/immunosuppressive agent conjugates.

In certain preferred embodiments, the cytotoxic agent is selected from the group consisting of an enediyne, a lexitropsin, a duocarmycin, a taxane, a puromycin, a dolastatin, a maytansinoid, and a vinca alkaloid. In certain, more specific embodiments, the cytotoxic agent is paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretastatin, calicheamicin, maytansine, DM-1, auristatin E, AEB, AEVB, AEFP, MMAE, or netropsin. The structures of AEB, AEVB, AEFP and MMAE are depicted in Section 3.1, *infra*.

In other preferred embodiments, the cytotoxic agent of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is an anti-tubulin agent. In more specific embodiments, the cytotoxic agent is selected from the group consisting of a vinca alkaloid, a podophyllotoxin, a taxane, a baccatin derivative, a cryptophysin, a maytansinoid, a combretastatin, and a dolastatin. In more specific embodiments, the

cytotoxic agent is vincristine, vinblastine, vindesine, vinorelbine, VP-16, camptothecin, paclitaxel, docetaxel, epithilone A, epithilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, maytansine, DM-1, AEFP, auristatin E, AEB, AEVB, AEFP, MMAE or eleutherobin.

In a specific embodiment, the cytotoxic agent of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is MMAE. In another specific embodiment, the cytotoxic agent of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is AEFP.

In specific embodiments, the anti-CD30 antibody of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is conjugated to the cytotoxic agent via a linker, wherein the linker is peptide linker. In specific embodiments, the anti-CD30 antibody of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is conjugated to the cytotoxic agent via a linker, wherein the linker is a val-cit linker, a phe-lys linker, a hydrazone linker, or a disulfide linker. In certain embodiments, the anti-CD30 antibody of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is conjugated to the cytotoxic agent via a peptide linker.

In certain embodiments, the conjugate of the invention is anti-CD30-valine-citrulline-MMAE (anti-CD30-val-citMMAE or anti-CD30-vcMMAE) or anti-CD30-valine-citrulline-AEFP (anti-CD30-val-citAEFP or anti-CD30-vcAEFP). In specific embodiments, the conjugate of the invention is AC10-valine-citrulline-MMAE (AC10-val-citMMAE or AC10-vcMMAE) or AC10-valine-citrulline-AEFP (AC10-val-citAEFP or AC10-vcAEFP).

In other embodiments, the conjugate of the invention is anti-CD30-phenylalanine-lysine-MMAE (anti-CD30-phe-lysMMAE or anti-CD30-fkMMAE) or anti-CD30-phenylalanine-lysine-AEFP (anti-CD30-phe-lysAEFP or anti-CD30-fkAEFP). In specific embodiments, the conjugate of the invention is AC10-phenylalanine-lysine-MMAE (AC10-phe-lysMMAE or AC10-fkMMAE) or AC10-phenylalanine-lysine-AEFP (AC10-phe-lysAEFP or AC10-fkAEFP).

Thus, in a specific embodiment, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for

said treatment, a pharmaceutical composition comprising (a) cAC10-val-cit-MMAE; and (b) a pharmaceutically acceptable carrier.

In another specific embodiment, the present invention provides methods for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) cAC10-val-cit-AEFP; and (b) a pharmaceutically acceptable carrier.

In certain embodiments, the anti-CD30 antibody of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is conjugated to the cytotoxic agent via a linker, wherein the linker is hydrolyzable at a pH of less than 5.5. In a specific embodiment the linker is hydrolyzable at a pH of less than 5.0.

In certain embodiments, the anti-CD30 antibody of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is conjugated to the cytotoxic agent via a linker, wherein the linker is cleavable by a protease. In a specific embodiment, the protease is a lysosomal protease. In other specific embodiments, the protease is, *inter alia*, a membrane-associated protease, an intracellular protease, or an endosomal protease.

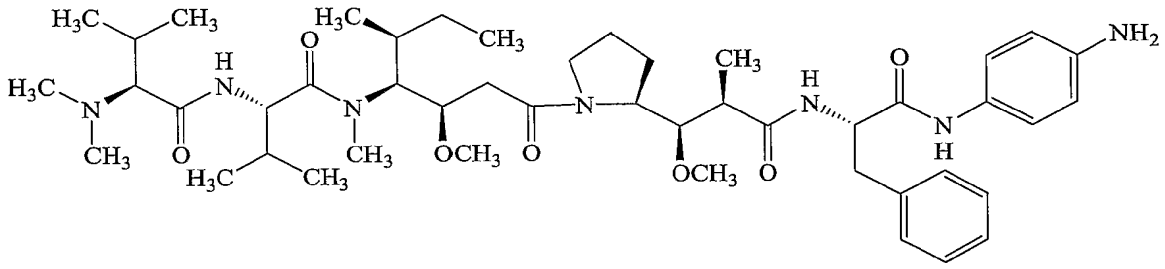
In certain embodiments, the anti-CD30 antibody of the invention is a monoclonal antibody, a humanized chimeric antibody, a chimeric antibody, a humanized antibody, a glycosylated antibody, a multispecific antibody, a human antibody, a single-chain antibody, a Fab fragment, a F(ab') fragment, a $F(ab')_2$ fragment, a Fd, a single-chain Fv, a disulfide-linked Fv, a fragment comprising a V_L domain, or a fragment comprising a V_H domain. In certain embodiments, the anti-CD30 antibody of an anti-CD30 antibody-cytotoxic agent conjugate of the invention is a polypeptide that binds specifically to CD30. In certain embodiments, the antibody is a bispecific antibody. In other embodiments, the antibody is not a bispecific antibody.

In certain embodiments, the anti-CD30 antibody is radioactively labeled. In certain embodiments, the anti-CD30 antibody of the anti-CD30 antibody-cytotoxic agent conjugate is radioactively labeled. In specific embodiments, the radioacive label is ^{90}Y, ^{111}In, ^{211}At, ^{131}I, ^{212}Bi, ^{213}Bi, ^{225}Ac, ^{186}Re, ^{188}Re, ^{109}Pd, ^{67}Cu, ^{77}Br, ^{105}Rh, ^{198}Au, ^{199}Au or ^{212}Pb.

Immunological disorders encompassed by the methods of the present invention are Th_2-lymphocyte related disorders (*e.g.*, atopic dermatitis, systemic lupus erythematosus ("SLE"), atopic asthma, rhinoconjunctivitis, allergic rhinitis, Omenn's syndrome, systemic sclerosis, and chronic graft versus host disease); Th_1 lymphocyte-related disorders (*e.g.*, rheumatoid arthritis, multiple sclerosis, psoriasis, Sjorgren's syndrome, Hashimoto's thyroiditis, Grave's disease, primary biliary cirrhosis, Wegener's granulomatosis, tuberculosis and acute graft versus host disease); viral infection-related disorders (*e.g.*, Epstein-Barr virus, human immunodeficiency virus, human T leukemia virus, hepatitis B virus, and measles virus infections); and activated B lymphocyte-related disorders (*e.g.*, systemic lupus erythematosus, Goodpasture's syndrome, rheumatoid arthritis, and type I diabetes).

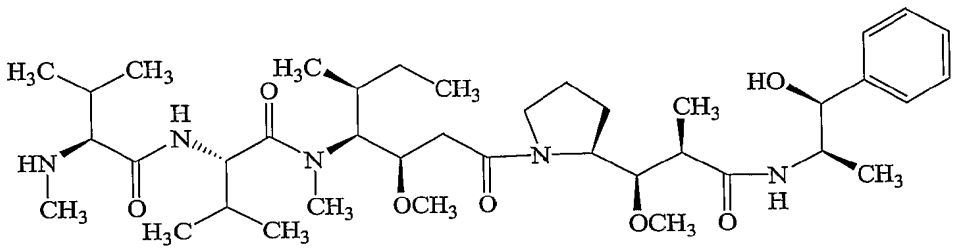
3.1 ABBREVIATIONS

The abbreviation "AEFP" refers to dimethylvaline-valine-dolaisoleuine-dolaproine-phenylalanine-p-phenylenediamine, the auristatin E derivative depicted below:



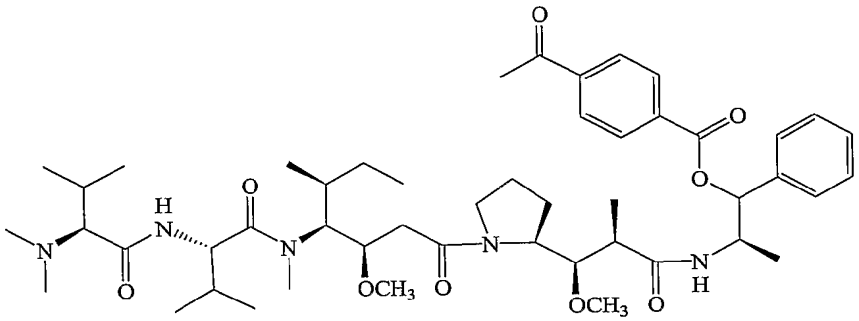
AEFP

The abbreviation "MMAE" refers to monomethyl auristatin E, the auristatin E derivative depicted below:



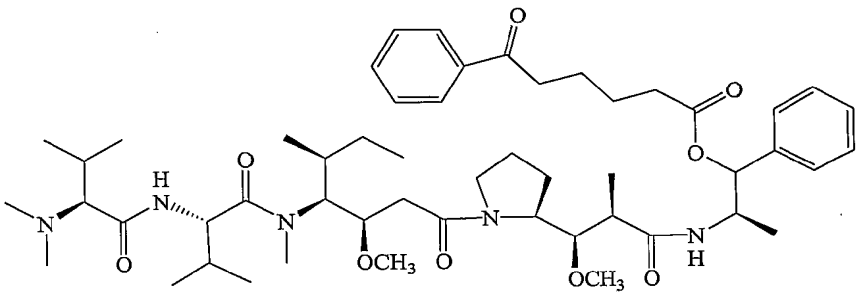
MMAE

The abbreviation "AEB" refers to an ester produced by reacting auristatin E with paraacetyl benzoic acid, the structure of which is depicted below:



AEB

The abbreviation "AEVB" refers to an ester produced by reacting auristatin E with benzoylvaleric acid, the structure of which is depicted below:



AEVB

The abbreviations "fk" and "phe-lys" refer to the linker phenylalanine-lysine.

The abbreviations "vc" and "val-cit" refer to the linker valine-citrulline.

4. BRIEF DESCRIPTION OF THE FIGURES

FIG. 1. Expression of CD30 on Jurkat T cells: The Jurkat T cell line was examined for the expression of CD3, CD4, CD28, and CD30 by flow cytometric analysis.

FIG. 2. Effect of anti-CD30 on the proliferation of Jurkat T cell: Jurkat T cells were incubated with graded doses of a chimeric AC10 (cAC10) anti-CD30 mAb with (XL cAC10) or without (cAC10) the presence of a secondary cross-linking goat anti-human (GAH) Fcγ specific Ab. Proliferation was assessed by a pulse of tritiated thymidine (^{3}H-TdR) during the last 4 hours of a 72 hour incubation.

FIG. 3. Secondary cross-linking of anti-CD30 mAb on the Jurkat T cells inhibited proliferation: Jurkat T cells were incubated with graded doses of the AC10 or HeFi-1 mAbs in the presence of a secondary cross-linking goat anti-mouse (GAM) Fcγ specific Ab at different primary to secondary Ab ratios as indicated in the figure.

Proliferation was assessed by a pulse of ^{3}H-TdR during the last 4 hours of a 48-hour incubation.

FIG. 4. Secondary cross-linking of anti-CD30 mAb on the Jurkat T cells induced apoptosis: Jurkat T cells were treated with 0.2 μg/ml of either AC10 or HeFi-1 cross-linked by 0.8 μg/ml of a GAM secondary Ab. Cell cycle disposition and DNA sysnthesis were detected by propidium iodide (PI) and anti-bromodeoxyuridine (BrdU) staining after 24 and 48 hours of treatment.

FIG. 5. Secondary cross-linking of anti-CD30 mAb on the Jurkat T cells induced apoptosis: Jurkat T cells were treated with graded doses of either AC10 or HeFi-1 cross-linked by a GAM secondary Ab at a primary to secondary Ab concentration ratio of 1:4. Cell cycle disposition and DNA synthesis were detected by PI and anti-BrdU staining after 24 and 48 hours of treatment.

FIG. 6. Anti-CD3 mAb enhanced anti-CD30-induced apoptosis in Jurkat T cells: Jurkat T cells were treated with AC10, the anti-CD3 mAb OKT3, or a combination of both mAbs at graded doses as indicated in the figure. Four-fold excess of a GAM secondary Ab was used to cross-link the primary mAbs. Cell cycle disposition and DNA sysnthesis were detected by PI and anti-BrdU staining after 24 and 48 hours of treatment.

FIG. 7. Detection of anti-CD30-induced apoptosis in Jurkat T cells by Annexin V binding: Jurkat T cells were treated with AC10, HeFi-1, the anti-CD3 mAb OKT3, or a combination of AC10 or HeFi-1 and OKT3. Anti-CD30 and anti-CD3 mAbs were used at 2 μg/ml. GAM secondary Ab was used in 10-fold excess to cross-link the primary mAbs. Binding of FITC-conjugated Annexin V enabled the detection of cells undergoing apoptosis. Membrane permeability to PI was used to detect dead cells that had lost membrane integrity. In the figure, Annexin V^{-}/PI^{-} events represent live cells (lower left quadrant), Annexin V^{+}/PI^{-} events represent apoptotic cells (lower right quadrant), and Annexin V^{+}/PI^{+} events represent dead cells (upper right quadrant). Numbers outside of the density plots denote the percentage of cells present in each of the quadrants.

FIG. 8. Chemical structures of the antibody drug conjugates (ADCs) cAC10-vcMMAE, cAC10-fkMMAE, cAC10-vcAEFP, and cAC10-fkAEFP.

FIG. 9. Growth inhibitory effect of the cAC10-vcMMAE conjugate on the proliferation of Jurkat T cells: Graded doses of the cAC10-vcMMAE conjugate or a non-binding control IgG (cIgG)-vcMMAE conjugate were added to Jurkat T cells at the initiation of culture. Cells were exposed to the ADCs continuously for a total of 96 hr. Proliferation was assessed by a pulse of ^{3}H-TdR during the last 16 hours of incubation.

FIG. 10. CD30 induction on activated normal peripheral blood mononuclear cells (PBMC): PBMC from normal donors were stimulated with either anti-CD3, anti-CD3 + anti-CD28, or in medium alone (negative control). On days 0, 2, 4, 6, and 8 cells were harvested and expression of CD30 was determined on both CD4 cells (shown) and CD8 cells (not shown) by multi-color flow cytometric analysis.

FIG. 11. Growth inhibitory effects of cAC10 ADCs on activated normal human PBMC: PBMC from normal donors were activated with anti-CD3 + anti-CD8 mAbs as described in FIG. 10. Graded doses of different ADCs as indicated in the figure were added to the cells at the initiation of culture. Cells were exposed to the ADCs continuously for a total of 48 or 72 hr. Proliferation was assessed by incorporation of ^{3}H-TdR during the last 16 hours of incubation.

FIG. 12. Induction of CD30 on memory and naïve T cells: Peripheral blood T lymphocytes, memory T lymphocytes, and naïve T lymphocytes were enriched from PBMC using immuno-selection. T cells were then activated by anti-CD3 + anti-CD28 mAbs for 72 hr in the presence of both recombinant human interleukin (rhIL)-2 and rhIL-4. Expression of CD30 on $CD4^{+}$ and $CD8^{+}$ cells was then assessed by flow cytometry.

FIG. 13. Growth inhibitory effects of cAC10 ADCs on activated memory and naïve T lymphocytes: Memory and naïve T cells enriched from PBMC were induced

to express CD30 as described in FIG. 12. After 72 hr of induction, T cells were harvested and treated with graded doses of different ADCs as indicated in the figure in the presence of rhIL-2. Cells were exposed to the ADCs continuously for an additional 48 or 72 hr. Proliferation was assessed by incorporation of ^{3}H-TdR during the last 16 hours of incubation.

FIG. 14. CD30 induction on T cells stimulated by allogeneic cells: CD4-enriched PBMC were stimulated with successive cycles of irradiated allogeneic Burkitt's lymphoma Daudi cells. Expression of CD30 on CD4 cells was determined by flow cytometric analysis 3-5 days and 7-9 days after the addition of the allogeneic stimulator cells.

FIG. 15. Generation of T lymphocyte clones: a schematic to summarize the generation of T cell clones from PBMC.

FIG. 16. Phenotype of T lymphocyte clones: Ten independent T lymphocyte clones isolated from 3 different normal donors according to the scheme depicted in FIG. 15 were examined for their surface expression of CD3, CD4, CD8, CD28, and CD30 by flow cytometric analysis. Histograms from two representative clones are shown, and the levels of receptor expression in all 10 clones indicated by the mean fluorescence intensities obtained from flow cytometric analysis were tabulated.

FIG. 17. Cytokine expression by T lymphocyte clones: T lymphocyte clones depicted in FIG. 16 were examined for their ability of express IL-2, 4, 5, 13, IFNγ, and TNFα by flow cytometric analysis. Histograms from two representative clones are shown, and the levels of cytokine expression in all 10 clones indicated by the mean fluorescence intensities obtained from flow cytometric analysis were tabulated. T lymphocytes clones were also assigned to different subsets based on cytokine expression in the bottom row of the table

FIG. 18. Upregulation of CD30 upon stimulation of T lymphocyte clones: A resting Th_2 clone and a resting Tc_2 clone representing the panel shown in FIG. 16 and 17 were stimulated with phytohemaggutinin (PHA), irradiated feeder cells, IL-2, and IL-4. CD25 and CD30 expression on days 0, 2, 4, and 7 were determined by flow cytometric analysis.

FIG. 19. Growth inhibitory effects of cAC10 ADCs on the T lymphocyte clone 40E10: 40E10 cells were induced to express CD30 as described in FIG. 18. At the peak of CD30 expression on day 2 of activation, cells were harvested and treated with graded doses of different ADCs as indicated in the figure in the presence of rhIL-2 and rhIL-4. Cells were exposed to the ADCs continuously for an additional 48 or 72 hr. Proliferation was assessed by incorporation of ^{3}H-TdR during the last 16 hours of incubation.

FIG. 20. Growth inhibitory effects of cAC10 ADCs on the T lymphocyte clone 40H7: 40H7 cells were induced to express CD30 as described in FIG. 18. At the peak of CD30 expression on day 2 of activation, cells were harvested and treated with graded doses of different ADCs as indicated in the figure in the presence of rhIL-2 and rhIL-4. Cells were exposed to the ADCs continuously for an additional 48 or 72 hr. Proliferation was assessed by a pulse of ^{3}H-TdR during the last 16 hours of incubation.

FIG. 21. Inhibition of proliferation in T cell clones induced by cAC10 ADCs was accompanied by apoptosis induction: Clone 40E10 and 40H7 were incubated with 1 μg/ml of different ADCs as indicated in the figure. After 48 hr of incubation, Annexin V binding and permeability of PI were used to assess the extent of apoptosis induction.

FIG. 22. Growth inhibitory effect of cAC10 ADCs on the T lymphocyte clones 3.27.2 and 4.01.1: 3.27.2 and 4.01.1 cells were induced to express CD30 as described in FIG. 18. At the peak of CD30 expression on day 2 of activation, cells were harvested and treated with graded doses of different ADCs as indicated in the figure in the

presence of rhIL-2. Cells were exposed to the ADCs continuously for an additional 72 hr. Proliferation was assessed by a pulse of ^{3}H-TdR during the last 4 hours of incubation.

FIG. 23. A summary of the efficacies of cAC10 ADCs to inhibit the proliferation of CD30^{+} T lymphocyte clones and activated normal T lymphocytes.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the use of proteins that bind to CD30 and induce CD30 signaling in a lymphocyte and/or exert a cytostatic or cytotoxic effect on activated lymphocytes to treat or prevent immunological disorders. The invention further relates to proteins that compete with AC10 or HeFi-1 for binding to CD30 and induce CD30 signaling in a lymphocyte and/or exert a cytostatic or cytotoxic effect on activated lymphocytes. In one embodiment, the protein is an antibody. In a preferred mode of the embodiment, the antibody is AC10 or HeFi-1, most preferably a humanized or chimeric form of AC10 or HeFi-1.

In certain embodiments, the anti-CD30 antibodies of the present invention are capable of inducing apoptosis or growth arrest of activated lymphocytes as monospecific antibodies, in the absence of conjugation to cytotoxic reagents (*e.g.*, small molecules, toxins, radioactive isotopes), and/or in the absence of cells other than the CD30-expressing lymphocytes (*e.g.*, in the absence of effector cells such as natural killer cells). Without being bound by any theory, the present inventors believe that such antibodies induce apoptosis or growth arrest by signaling through the CD30 pathway.

The invention further relates to the use of proteins encoded by and nucleotide sequences of AC10 and HeFi-1 genes to treat or prevent immunological disorders. The invention further relates to fragments and other derivatives and analogs of such AC10 and HeFi-1 proteins. Nucleic acids encoding such fragments or derivatives are also within the scope of the invention. Production of the foregoing proteins, *e.g.*, by recombinant methods, is provided.

The invention also relates to the use of AC10 and HeFi-1 proteins and derivatives including fusion/chimeric proteins which are functionally active, *i.e.*, which

are capable of displaying binding to CD30 and exerting a cytostatic or cytotoxic effect on activated lymphocytes, to treat or prevent immunological disorders.

Antibodies to CD30 whose use is encompassed by the invention include human, chimeric or humanized antibodies, and such antibodies conjugated to cytotoxic agents such as chemotherapeutic drugs.

The invention further relates to methods of treating or preventing immunological disorders comprising administering a composition comprising a protein or nucleic acid of the invention alone or in combination with a cytotoxic agent, including but not limited to a chemotherapeutic drug.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

5.1 PROTEINS OF THE INVENTION

The present invention encompasses the use of proteins, including but not limited to antibodies, that bind to CD30 and exert cytostatic and/or cytotoxic effects on activated lymphocytes, for the treatment of an immunological disorder. The invention further relates to the use of proteins that compete with AC10 or HeFi-1 for binding to CD30 and exert a cytostatic or cytotoxic effect on activated lymphocytes for the treatment of immunological disorders.

The present invention further encompasses the use of proteins comprising a CDR of HeFi-1 (SEQ ID NO:20, SEQ ID NO:22; SEQ ID NO:24; SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:32) or AC10 (SEQ ID NO:4; SEQ ID NO:6; SEQ ID NO:8; SEQ ID NO:12; SEQ ID NO:14; or SEQ ID NO:16) for the treatment of an immunological disorder.

The present invention further encompasses the use of proteins comprising a variable region of HeFi-1 (SEQ ID NO:18 or SEQ ID NO:26) or AC10 (SEQ ID NO:2 or SEQ ID NO:10) for treating an immunological disorder. A table indicating the region of AC10 or HeFi-1 to which each SEQ ID NO corresponds to is provided below:

Table 1

MOLECULE	NUCLEOTIDE OR AMINO ACID	SEQ ID NO
AC10 Heavy Chain Variable Region	Nucleotide	1
AC10 Heavy Chain Variable Region	Amino Acid	2
AC10 Heavy Chain-CDR1(H1)	Nucleotide	3
AC 10 Heavy Chain-CDR1(H1)	Amino Acid	4
AC 10 Heavy Chain-CDR2(H2)	Nucleotide	5
AC 10 Heavy Chain-CDR2(H2)	Amino Acid	6
AC 10 Heavy Chain-CDR3(H3)	Nucleotide	7
AC 10 Heavy Chain-CDR3(H3)	Amino Acid	8
AC 10 Light Chain Variable Region	Nucleotide	9
AC 10 Light Chain Variable Region	Amino Acid	10
AC 10 Light Chain-CDR1(L1)	Nucleotide	11
AC 10 Light Chain-CDR1(L1)	Amino Acid	12
AC 10 Light Chain-CDR2(L2)	Nucleotide	13
AC 10 Light Chain-CDR2(L2)	Amino Acid	14
AC 10 Light Chain-CDR3(L3)	Nucleotide	15
AC 10 Light Chain-CDR3(L3)	Amino Acid	16
HeFi-1 Heavy Chain Variable Region	Nucleotide	17
HeFi-1 Heavy Chain Variable Region	Amino Acid	18
HeFi-1 Heavy Chain-CDR1(H1)	Nucleotide	19
HeFi-1 Heavy Chain-CDR1(H1)	Amino Acid	20
HeFi-1 Heavy Chain-CDR2(H2)	Nucleotide	21
HeFi-1 Heavy Chain-CDR2(H2)	Amino Acid	22
HeFi-1 Heavy Chain-CDR3(H3)	Nucleotide	23
HeFi-1 Heavy Chain-CDR3(H3)	Amino Acid	24
HeFi-1 Light Chain Variable Region	Nucleotide	25
HeFi-1 Light Chain Variable Region	Amino Acid	26
HeFi-1 Light Chain-CDR1(L1)	Nucleotide	27

MOLECULE	NUCLEOTIDE OR AMINO ACID	SEQ ID NO
HeFi-1 Light Chain-CDR1(L1)	Amino Acid	28
HeFi-1 Light Chain-CDR2(L2)	Nucleotide	29
HeFi-1 Light Chain-CDR2(L2)	Amino Acid	30
HeFi-1 Light Chain-CDR3(L3)	Nucleotide	31
HeFi-1 Light Chain-CDR3(L3)	Amino Acid	32

The present invention further comprises the use of functional derivatives or analogs of AC10 and HeFi-1 for treating immunological disorders. As used herein, the term "functional" in the context of a peptide or protein of the invention indicates that the peptide or protein is 1) capable of binding to CD30 and 2) induces CD30 signaling in a lymphocyte and/or exerts a cytostatic and/or cytotoxic effect on activated lymphocytes.

Generally, antibodies suitable for practicing the methods of the present invention immunospecifically bind CD30 and induce CD30 signaling in a lymphocyte and/or exert cytostatic and cytotoxic effects on activated lymphocytes. Antibodies suitable for practicing the methods of the invention are preferably monoclonal, and may be multispecific, human, humanized or chimeric antibodies, single chain antibodies, Fab fragments, F(ab') fragments, fragments produced by a Fab expression library, and CD30 binding fragments of any of the above. The term "antibody," as used herein, refers to immunoglobulin molecules and immunologically active portions of immunoglobulin molecules, *i.e.*, molecules that contain an antigen binding site that immunospecifically binds CD30. The immunoglobulin molecules of the invention can be of any type (*e.g.*, IgG, IgE, IgM, IgD, IgA and IgY), class (*e.g.*, IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2) or subclass of immunoglobulin molecule.

In certain embodiments of the invention, the antibodies are human antigen-binding antibody fragments of the present invention and include, but are not limited to, Fab, Fab' and $F(ab')_2$, Fd, single-chain Fvs (scFv), single-chain antibodies, disulfide-linked Fvs (sdFv) and fragments comprising either a V_L or V_H domain. Antigen-binding antibody fragments, including single-chain antibodies, may comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, CH1, CH2, CH3 and CL domains. Also included in the invention are antigen-

binding fragments also comprising any combination of variable region(s) with a hinge region, CH1, CH2, CH3 and CL domains. Preferably, the antibodies are human, murine (*e.g.*, mouse and rat), donkey, sheep, rabbit, goat, guinea pig, camelid, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries, from human B cells, or from animals transgenic for one or more human immunoglobulin, as described *infra* and, for example in U.S. Patent No. 5,939,598 by Kucherlapati *et al.*

The antibodies suitable for practicing the methods of the present invention may be monospecific, bispecific, trispecific or of greater multispecificity. Multispecific antibodies may be specific for different epitopes of CD30 or may be specific for both CD30 as well as for a heterologous protein. *See, e.g.*, PCT publications WO 93/17715; WO 92/08802; WO 91/00360; WO 92/05793; Tutt, *et al.*, 1991, J. Immunol. 147:60-69; U.S. Patent Nos. 4,474,893; 4,714,681; 4,925,648; 5,573,920; 5,601,819; Kostelny *et al.*, 1992, J. Immunol. 148:1547-1553. Multispecific antibodies, including bispecific and trispecific antibodies, useful for practicing the present invention are antibodies that immunospecifically bind to both CD30 (including but not limited to antibodies that have the CDRs and/or heavy chains of the monoclonal antibodies Ki-2, Ki-4, Ki-5, Ki-7, Ber-H2, HRS-1, HRS-4, Ki-1, Ki-6, M67, Ki-3, M44, HeFi-1, and AC10) and a lymphocyte surface receptor or receptor complex, such as an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin (C-type, S-type, or I-type), or a complement control protein. Examples of such molecules, and antibodies against such molecules from which a bispecific antibody can be derived, are provided in Section 5.12.2, *infra*. In a preferred embodiment, the binding of the portion of the multispecific antibody to the lymphocyte cell surface molecule or molecular complex enhances the cytotoxic or cytostatic effect of the anti-CD30 antibody by delivering a cytostatic or cytotoxic signal to the activated lymphocytes.

In certain specific embodiment of the present invention, the anti-CD30 antibody is an agonistic antibody. In another specific embodiment of the present

invention, the anti-CD30 antibody is not a non-agonistic antibody. In another specific embodiment, the anti-CD30 antibody does not block binding of CD30 ligand to CD30.

Antibodies useful in the present methods may be described or specified in terms of the particular CDRs they comprise. In certain embodiments antibodies of the invention comprise one or more CDRs of AC10 and/or HeFi-1. The invention encompasses the use of an antibody or derivative thereof comprising a heavy or light chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs are from monoclonal antibody AC10 or HeFi-1, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody AC10 or HeFi-1, respectively, and in which said antibody or derivative thereof immunospecifically binds CD30 and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

In a specific embodiment, the invention encompasses the use of an antibody or derivative thereof for treating an immunological disorder, wherein the antibody comprises a heavy chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:4, 6, or 8 and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody AC10, and in which said antibody or derivative thereof immunospecifically binds CD30, and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

In a specific embodiment, the invention encompasses the use of an antibody or derivative thereof for the treatment of an immunological disorder, wherein the antibody comprises a heavy chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:20, 22 or 24 and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody HeFi-1, and in which said antibody or derivative thereof immunospecifically binds CD30, and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

In a specific embodiment, the invention encompasses the use of an antibody or derivative thereof for the treatment of an immunological disorder, wherein the

antibody comprises a light chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:12, 14 or 16, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody AC10, and in which said antibody or derivative thereof immunospecifically binds CD30, and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

In a specific embodiment, the invention encompasses using an antibody or derivative thereof for the treatment of an immunological disease, wherein the antibody comprises a light chain variable domain, said variable domain comprising (a) a set of three CDRs, in which said set of CDRs comprises SEQ ID NO:28, 30, or 32, and (b) a set of four framework regions, in which said set of framework regions differs from the set of framework regions in monoclonal antibody HeFi-1, and in which said antibody or derivative thereof immunospecifically binds CD30, and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

Additionally, antibodies that may be used in the methods of the present invention may also be described or specified in terms of their primary structures. Antibodies having at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% and most preferably at least 98% identity (as calculated using methods known in the art and described herein) to the variable regions and AC10 or HeFi-1 are also included in the present methods for treating immunological disorders. Antibodies useful in the methods of the present invention may also be described or specified in terms of their binding affinity to CD30. Preferred binding affinities include those with a dissociation constant or Kd less than 5 X 10^{-2} M, 10^{-2} M, 5 X 10^{-3} M, 10^{-3} M, 5 X 10^{-4} M, 10^{-4} M, 5 X 10^{-5} M, 10^{-5} M, 5 X 10^{-6} M, 10^{-6} M, 5 X 10^{-7} M, 10^{-7} M, 5 X 10^{-8} M, 10^{-8} M, 5 X 10^{-9} M, 10^{-9} M, 5 X 10^{-10} M, 10^{-10} M, 5 X 10^{-11} M, 10^{-11} M, 5 X 10^{-12} M, 10^{-12} M, 5 X $^{-13}$ M, 10^{-13} M, 5 X 10^{-14} M, 10^{-14} M, 5 X 10^{-15} M, or 10^{-15} M.

The antibodies of the invention, *i.e.*, antibodies that are useful for treating immunological disorders, include derivatives that are modified, *i.e,* by the covalent attachment of any type of molecule to the antibody such that covalent attachment does not prevent the antibody from binding to CD30 or from exerting a cytostatic or cytotoxic

effect on activated lymphocytes. For example, but not by way of limitation, the antibody derivatives include antibodies that have been modified, *e.g.*, by glycosylation, acetylation, pegylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to a cellular ligand or other protein, *etc.* Any of numerous chemical modifications may be carried out by known techniques, including, but not limited to specific chemical cleavage, acetylation, formylation, metabolic synthesis of tunicamycin, *etc.* Additionally, the derivative may contain one or more non-classical amino acids.

The antibodies that may be used in the treatment of immunological disorders may be generated by any suitable method known in the art. Polyclonal antibodies to CD30 can be produced by various procedures well known in the art. For example, CD30 can be administered to various host animals including, but not limited to, rabbits, mice, rats, *etc.* to induce the production of sera containing polyclonal antibodies specific for the protein. Various adjuvants may be used to increase the immunological response, depending on the host species, and include but are not limited to, Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and corynebacterium parvum. Such adjuvants are also well known in the art.

Monoclonal antibodies can be prepared using a wide variety of techniques known in the art including the use of hybridoma, recombinant, and phage display technologies, or a combination thereof. For example, monoclonal antibodies can be produced using hybridoma techniques including those known in the art and taught, for example, in Harlow *et al.*, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratory Press, 2nd ed., 1988); Hammerling, *et al.*, in: Monoclonal Antibodies and T-Cell Hybridomas 563-681 (Elsevier, N.Y., 1981) (said references incorporated by reference in their entireties). The term "monoclonal antibody" as used herein is not limited to antibodies produced through hybridoma technology. The term "monoclonal antibody" refers to an antibody that is derived from a single clone, including any eukaryotic, prokaryotic, or phage clone, and not the method by which it is produced.

Methods for producing and screening for specific antibodies using hybridoma technology are routine and well known in the art. In a non-limiting example, mice can be immunized with CD30 or a cell expressing CD30 or a fragment or derivative thereof. Once an immune response is detected, *e.g.*, antibodies specific for CD30 are detected in the mouse serum, the mouse spleen is harvested and splenocytes isolated. The splenocytes are then fused by well known techniques to any suitable myeloma cells, for example cells from cell line SP20 available from the ATCC. Hybridomas are selected and cloned by limited dilution. The hybridoma clones are then assayed by methods known in the art for cells that secrete antibodies capable of binding CD30 and exerting a cytotoxic or cytostatic effect on activated lymphocytes. Ascites fluid, which generally contains high levels of antibodies, can be generated by injecting mice with positive hybridoma clones.

Antibody fragments which recognize specific epitopes may be generated by known techniques. For example, Fab and $F(ab')_2$ fragments of the invention may be produced by proteolytic cleavage of immunoglobulin molecules, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce $F(ab')_2$ fragments). $F(ab')_2$ fragments contain the variable region, the light chain constant region and the CH 1 domain of the heavy chain.

For example, antibodies useful in the methods of the present invention can also be generated using various phage display methods known in the art. In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the nucleic acid sequences encoding them. In a particular embodiment, such phage can be utilized to display antigen binding domains expressed from a repertoire or combinatorial antibody library (*e.g.*, human or murine). In phage display methods, functional antibody domains are displayed on the surface of phage particles which carry the nucleic acid sequences encoding them. In particular, DNA sequences encoding V_H and V_L domains are amplified from animal cDNA libraries (*e.g.*, human or murine cDNA libraries of lymphoid tissues). The DNA encoding the V_H and V_L domains are recombined together with an scFv linker by PCR and cloned into a phagemid vector (*e.g.*, p CANTAB 6 or pComb 3 HSS). The vector is electroporated in *E. coli* and the *E. coli* is infected with helper phage. Phage used in these methods are typically filamentous

phage including fd and Ml3 binding domains expressed from phage with Fab, Fv or disulfide stabilized Fv antibody domains recombinantly fused to either the phage gene III or gene VIII protein. Phage expressing an antigen binding domain that binds to CD30 or an AC10 or HeFi- binding portion thereof can be selected or identified with antigen *e.g.*, using labeled antigen or antigen bound or captured to a solid surface or bead. Examples of phage display methods that can be used to make the antibodies of the present invention include those disclosed in Brinkman *et al.*, 1995, J. Immunol. Methods 182:41-50; Ames *et al.*, 1995, J. Immunol. Methods 184:177-186; Kettleborough *et al.*, 1994, Eur. J. Immunol. 24:952-958; Persic *et al.*, 1997, Gene 187:9-18; Burton *et al.*, 1994, Advances in Immunology, 191-280; PCT Application No. PCT/GB91/O1 134; PCT Publications WO 90/02809; WO 91/10737; WO 92/01047; WO 92/18619; WO 93/1 1236; WO 95/15982; WO 95/20401; and U.S. Patent Nos. 5,698,426; 5,223,409; 5,403,484; 5,580,717; 5,427,908; 5,750,753; 5,821,047; 5,571,698; 5,427,908; 5,516,637; 5,780,225; 5,658,727; 5,733,743 and 5,969,108; each of which is incorporated herein by reference in its entirety.

As described in the above references, after phage selection, the antibody coding regions from the phage can be isolated and used to generate whole antibodies, including human antibodies, or any other desired antigen binding fragment, and expressed in any desired host, including mammalian cells, insect cells, plant cells, yeast, and bacteria, *e.g.*, as described in detail below. For example, techniques to recombinantly produce Fab, Fab' and $F(ab')_2$ fragments can also be employed using methods known in the art such as those disclosed in PCT publication WO 92/22324; Mullinax *et al.*, BioTechniques 1992, 12(6):864-869; and Sawai *et al.*, 1995, AJRI 34:26-34; and Better *et al.*, 1988, Science 240:1041-1043 (said references incorporated by reference in their entireties).

Examples of techniques which can be used to produce single-chain Fvs and antibodies include those described in U.S. Patents 4,946,778 and 5,258,498; Huston *et al.*, 1991, Methods in Enzymology 203:46-88 ; Shu *et al.*, 1993, PNAS 90:7995-7999; and Skerra *et al.*, 1988, Science 240:1038-1040 . For some uses, including *in vivo* use of antibodies in humans and *in vitro* proliferation or cytotoxicity assays, it is preferable to use chimeric, humanized, or human antibodies. A chimeric antibody is a molecule in

which different portions of the antibody are derived from different animal species, such as antibodies having a variable region derived from a murine monoclonal antibody and a human immunoglobulin constant region. Methods for producing chimeric antibodies are known in the art. *See e.g.*, Morrison, Science, 1985, 229:1202 ; Oi *et al.*, 1986, BioTechniques 4:214; Gillies *et al.*, 1989, J. Immunol. Methods 125:191-202; U.S. Patent Nos. 5,807,715; 4,816,567; and 4,816,397, which are incorporated herein by reference in their entirety. Humanized antibodies are antibody molecules from non-human species antibodies that bind the desired antigen having one or more CDRs from the non-human species and framework and constant regions from a human immunoglobulin molecule. Often, framework residues in the human framework regions will be substituted with the corresponding residue from the CDR donor antibody to alter, preferably improve, antigen binding. These framework substitutions are identified by methods well known in the art, *e.g.*, by modeling of the interactions of the CDR and framework residues to identify framework residues important for antigen binding and sequence comparison to identify unusual framework residues at particular positions. (*See*, *e.g.*, Queen *et al.*, U.S. Patent No. 5,585,089; Riechmann *et al.*, 1988, Nature 332:323, which are incorporated herein by reference in their entireties.) Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 9 1/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, Molecular Immunology, 1991, 28(4/5):489-498; Studnicka *et al.*, 1994, Protein Engineering 7(6):805-814; Roguska. *et al.*, 1994, PNAS 91:969-973), and chain shuffling (U.S. Patent No. 5,565,332).

Completely human antibodies are particularly desirable for the therapeutic treatment of human patients. Human antibodies can be made by a variety of methods known in the art including phage display methods described above using antibody libraries derived from human immunoglobulin sequences. See also, U.S. Patent Nos. 4,444,887 and 4,716,111; and PCT publications WO 98/46645, WO 98/50433, WO 98/24893, WO 98/16654, WO 96/34096, WO 96/33735, and WO 91/10741; each of which is incorporated herein by reference in its entirety.

Human antibodies can also be produced using transgenic mice which express human immunoglobulin genes. For example, the human heavy and light chain

immunoglobulin gene complexes may be introduced randomly or by homologous recombination into mouse embryonic stem cells. The mouse heavy and light chain immunoglobulin genes may be rendered non-functional separately or simultaneously with the introduction of human immunoglobulin loci by homologous recombination. In particular, homozygous deletion of the JH region prevents endogenous antibody production. The modified embryonic stem cells are expanded and microinjected into blastocysts to produce chimeric mice. The chimeric mice are then bred to produce homozygous offspring which express human antibodies. The transgenic mice are immunized in the normal fashion with a selected antigen, *e.g.*, all or a portion of CD30. Monoclonal antibodies directed against the antigen can be obtained from the immunized, transgenic mice using conventional hybridoma technology. The human immunoglobulin transgenes harbored by the transgenic mice rearrange during B cell differentiation, and subsequently undergo class switching and somatic mutation. Thus, using such a technique, it is possible to produce therapeutically useful IgG, IgA, IgM and IgE antibodies. For an overview of this technology for producing human antibodies, *see*, Lonberg and Huszar, 1995, Int. Rev. Immunol. 13:65-93. For a detailed discussion of this technology for producing human antibodies and human monoclonal antibodies and protocols for producing such antibodies, *see*, *e.g.*, PCT publications WO 98/24893; WO 92/01047; WO 96/34096; WO 96/33735; European Patent No. 0 598 877; U.S. Patent Nos. 5,413,923; 5,625,126; 5,633,425; 5,569,825; 5,661,016; 5,545,806; 5,814,318; 5,885,793; 5,916,771; and 5,939,598, which are incorporated by reference herein in their entirety. In addition, companies such as Abgenix, Inc. (Freemont, CA) and Medarex (Princeton, NJ) can be engaged to provide human antibodies directed against a selected antigen using technology similar to that described above.

Completely human antibodies which recognize a selected epitope can be generated using a technique referred to as "guided selection." In this approach a selected non-human monoclonal antibody, *e.g.*, a mouse antibody, is used to guide the selection of a completely human antibody recognizing the same epitope. (Jespers *et al.*, 1994, Bio/technology 12:899-903).

Further, antibodies to CD30 can, in turn, be utilized to generate anti-idiotype antibodies that "mimic" proteins of the invention using techniques well known to

those skilled in the art. (*See, e.g.*, Greenspan & Bona, 1989, FASEB J. 7(5):437-444; and Nissinoff, 1991, J. Immunol. 147(8):2429-2438). Fab fragments of such anti-idiotypes can be used in therapeutic regimens to elicit an individual's own immune response against CD30 present on activated lymphocytes.

As alluded to above, proteins that are therapeutically or prophylactically useful against activated lymphocytes need not be antibodies. Accordingly, proteins of the invention may comprise one or more CDRs from an antibody that binds to CD30 and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic and/or cytostatic effect on activated lymphocytes. Preferably, a protein of the invention is a multimer, most preferably a dimer. As used herein, a "protein of the invention" is a protein, including but not limited to an antibody, that binds to CD30, and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

The invention also provides methods of treating immunological disorders using proteins, including but not limited to antibodies, that competitively inhibit binding of AC10 or HeFi-1 to CD30 as determined by any method known in the art for determining competitive binding, for example, the immunoassays described herein. In preferred embodiments, the protein competitively inhibits binding of AC10 or HeFi-1 to CD30 by at least 50%, more preferably at least 60%, yet more preferably at least 70%, and most preferably at least 75%. In other embodiments, the protein competitively inhibits binding of AC10 or HeFi-1 to CD30 by at least 80%, at least 85%, at least 90%, or at least 95%.

As discussed in more detail below, the present invention provides methods of treating immunological disorders using proteins, including antibodies, that bind to activated lymphocytes and exert a cytostatic or cytotoxic effect on the lymphocytes. The proteins can be administered either alone or in combination with other compositions in the prevention or treatment of immunological disorders. The proteins may further be recombinantly fused to a heterologous protein at the N– or C-terminus or chemically conjugated (including covalently and non-covalently conjugations) to cytotoxic agents, proteins or other compositions. For example, antibodies of the present invention may be recombinantly fused or conjugated to molecules useful as chemotherapeutics or toxins, or

comprise a radionuclide for use as a radio-therapeutic. *See, e.g.*, PCT publications WO 92/08495; WO 91/14438; WO 89/12624; U.S. Patent No. 5,314,995; and EP 396,387.

Proteins useful in the present methods may be produced recombinantly by fusing the coding region of one or more of the CDRs of an antibody of the invention in frame with a sequence coding for a heterologous protein. The heterologous protein may provide one or more of the following characteristics: added therapeutic benefits; promote stable expression; provide a means of facilitating high yield recombinant expression; or provide a multimerization domain.

In addition to proteins comprising one or more CDRs of an antibody that binds to CD30, and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on lymphocytes, proteins that are useful in the therapeutic methods of the invention may be identified using any method suitable for screening for protein-protein interactions. Initially, proteins are identified that bind to CD30, then their ability to exert a cytostatic or cytotoxic effect on activated lymphocytes can be determined. Among the traditional methods which can be employed are "interaction cloning" techniques which entail probing expression libraries with labeled CD30 in a manner similar to the technique of antibody probing of λgt11 libraries, *supra*. By way of example and not limitation, this can be achieved as follows: a cDNA clone encoding CD30 (or an AC10 or HeFi-1 binding domain thereof) is modified at the terminus by inserting the phosphorylation site for the heart muscle kinase (HMK) (Blanar & Rutter, 1992, Science 256:1014-1018). The recombinant protein is expressed in *E. coli* and purified on a GDP-affinity column to homogeneity (Edery *et al.*, 1988, Gene 74:517-525) and labeled using γ^{32}P-ATP and bovine heart muscle kinase (Sigma) to a specific activity of $1x10^8$ cpm/μg, and used to screen a human placenta λgt11 cDNA library in a "far-Western assay" (Blanar & Rutter, 1992, Science 256:1014-1018). Plaques which interact with the CD30 probe are isolated. The cDNA inserts of positive λ plaques are released and subcloned into a vector suitable for sequencing, such as pBluescript KS (Stratagene).

One method which detects protein interactions *in vivo*, the two-hybrid system, is described in detail for illustration purposes only and not by way of limitation. One version of this system has been described (Chien *et al.*, 1991, Proc. Natl. Acad. Sci. USA, 88:9578-9582) and is commercially available from Clontech (Palo Alto, CA).

Once a CD30-binding protein is identified, its ability (alone or when multimerized or fused to a dimerization or multimerization domain) to elicit a cytostatic or cytotoxic effect on activated lymphocytes is determined by the methods described in Section 5.7, *infra*.

Without limitation as to mechanism of action, a protein that binds to CD30 and induces CD30 signaling in a lymphocyte and/or exerts a cytostatic or cytotoxic effect on activated lymphocytes preferably has more than one CD30-binding site and therefore a capacity to cross link CD30 molecules on the surface of an activated lymphocyte. Proteins which bind to CD30 or compete for binding to CD30 with AC10 or HeFi-1 can acquire the ability to induce cytostatic or cytotoxic effects on activated lymphocytes if dimerized or multimerized. Where the CD30-binding protein is a monomeric protein, it can be expressed in tandem, thereby resulting in a protein with multiple CD30 binding sites. The CD30-binding sites can be separated by a flexible linker region. In another embodiment, the CD30-binding proteins can be chemically cross-linked, for example using gluteraldehyde, prior to administration. In a preferred embodiment, the CD30-binding region is fused with a heterologous protein, wherein the heterologous protein comprises a dimerization and multimerization domain. Prior to administration of the protein of the invention to a subject for the purpose of treating or preventing immunoglocial disorders, such a protein is subjected to conditions that allows formation of a homodimer or heterodimer. A heterodimer, as used herein, may comprise identical dimerization domains but different CD30-binding regions, identical CD30-binding regions but different dimerization domains, or different CD30-binding regions and dimerization domains.

Particularly preferred dimerization domains are those that originate from transcription factors.

In one embodiment, the dimerization domain is that of a basic region leucine zipper ("bZIP"). bZIP proteins characteristically possess two domains--a leucine zipper structural domain and a basic domain that is rich in basic amino acids, separated by a "fork" domain (C. Vinson *et al.*, 1989, Science, 246:911-916). Two bZIP proteins dimerize by forming a coiled coil region in which the leucine zipper domains dimerize.

Accordingly, these coiled coil regions may be used as fusion partners for proteins that will be useful in the therapeutic methods described herein.

Particularly useful leucine zipper domain are those of the yeast transcription factor GCN4, the mammalian transcription factor CCAAT/enhancer-binding protein C/EBP, and the nuclear transform in oncogene products, Fos and Jun (see Landschultz *et al.*, 1988, Science 240:1759-1764; Baxevanis and Vinson, 1993, Curr. Op. Gen. Devel., 3:278-285; and O'Shea *et al.*, 1989, Science, 243:538-542).

In another embodiment, the dimerization domain is that of a basic-region helix-loop-helix ("bHLH") protein (Murre *et al.*, 1989, Cell, 56:777-783). bHLH proteins are also composed of discrete domains, the structure of which allows them to recognize and interact with specific sequences of DNA. The helix-loop-helix region promotes dimerization through its amphipathic helices in a fashion analogous to that of the leucine zipper region of the bZIP proteins (Davis *et al.*, 1990 Cell, 60:733-746; Voronova and Baltimore, 1990 Proc. Natl. Acad. Sci. USA, 87:4722-4726). Particularly useful hHLH proteins are myc, max, and mac.

Heterodimers are known to form between Fos and Jun (Bohmann *et al.*, 1987, Science, 238:1386-1392), among members of the ATF/CREB family (Hai *et al.*,1989, Genes Dev., 3:2083-2090), among members of the C/EBP family (Cao *et al.*, 1991, Genes Dev., 5:1538-1552; Williams *et al.*, 1991, Genes Dev., 5:1553-1567; and Roman *et al.*, 1990, Genes Dev., 4:1404-1415), and between members of the ATF/CREB and Fos/Jun families Hai and Curran, 1991, Proc. Natl. Acad. Sci. USA, 88:3720-3724). Therefore, when a protein of the invention is administered to a subject as a heterodimer comprising different dimerization domains, any combination of the foregoing may be used.

In a preferred aspect, a proteins of the invention, including but not limited to an antibody-drug conjugate of the invention, is substantially purified (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects). In certain specific embodiments, the protein of the invention is 40% pure, more preferably about 50% pure, and most preferably about 60% pure. In certain specific embodiments, the protein of the invention is approximately 60-65%, 65-70%, 70-75%, 75-80%, 80-85%,

85-90%, 90-95%, or 95-98% pure. In another specific embodiment, the protein of the invention is approximately 99% pure.

5.1.1 ISOLATION OF AC10 or HeFi-1 GENES

The invention relates to the use of AC10 or HeFi-1 nucleic acids, e.g., for gene therapy of immunological disorders or for recombinant expression of an antibody molecule that can be use for the treatment of an immunological disorder. Accordingly, the invention provides purified nucleic acids consisting of at least 8 nucleotides (*i.e.*, a hybridizable portion) of an AC10 or HeFi-1 gene sequence; in other embodiments, the nucleic acids consist of at least 25 (contiguous) nucleotides, 50 nucleotides, 100, or 200 nucleotides of an AC10 or HeFi-1 sequence, or a full-length AC10 or HeFi-1 variable region coding sequence. In the same or other embodiments, the nucleic acids are smaller than 50, 75, 100, or 200 or 5000 nucleotides in length. Nucleic acids can be single or double stranded. The invention also relates to nucleic acids hybridizable to or complementary to the foregoing sequences or their reverse complements, and in particular, such nucleic acids that encode proteins that bind to CD30, compete with AC10 or HeFi-1 for binding to CD30, and/or increase the binding of CD30 ligand to CD30 by at least 45%, 50%, 60%, or 65%. In specific aspects, nucleic acids are provided which comprise a sequence complementary to at least 10, 25, 50, 100, or 200 nucleotides or the entire coding region of an AC10 or HeFi-1 variable region gene.

Nucleic acids encoding derivatives and analogs of AC10 or HeFi-1 proteins are additionally provided.

5.1.2 CLONING PROCEDURES

Specific embodiments for the cloning of an AC10 or HeFi-1 nucleic acid follow. In a specific embodiment, total RNA is isolated from a mAb AC10 or HeFi-1-producing hybridoma and polymerase chain reaction is used to amplify desired variable region sequences, using primers based on the sequences disclosed herein. By way of another example, mRNA is isolated from a mAb AC10 or HeFi-1-producing hybridoma, cDNA is made and ligated into an expression vector (*e.g.*, a bacteriophage derivative) such that it is capable of being expressed by the host cell into which it is then introduced.

Various screening assays can then be used to select for the expressed product. In one embodiment, selection is on the basis of hybridization to a labeled probe representing a portion of an AC10 or HeFi-1 gene or its RNA or a fragment thereof (Benton and Davis, 1977, Science 196:180; Grunstein and Hogness, 1975, Proc. Natl. Acad. Sci. U.S.A. 72:3961). Those DNA fragments with substantial homology to the probe will hybridize. It is also possible to identify the appropriate fragment by restriction enzyme digestion(s) and comparison of fragment sizes with those expected according to a known restriction map if such is available. Further selection can be carried out on the basis of the properties of the gene.

Alternatively, the presence of the desired gene may be detected by assays based on the physical, chemical, or immunological properties of its expressed product. For example, cDNA clones, or DNA clones which hybrid-select the proper mRNAs, can be selected and expressed to produce a protein that has, *e.g.*, similar or identical electrophoretic migration, isoelectric focusing behavior, proteolytic digestion maps, or functional activity, as known for an AC10 or HeFi-1 protein. For example, ability to bind CD30 can be detected in an ELISA (enzyme-linked immunosorbent assay)-type procedure.

An AC10 or HeFi-1 gene can also be identified by mRNA selection using nucleic acid hybridization followed by *in vitro* translation. In this procedure, fragments are used to isolate complementary mRNAs by hybridization. Functional assays (*e.g.*, binding to CD30, *etc.*) of the *in vitro* translation products of the isolated products of the isolated mRNAs identifies the mRNA and, therefore, the complementary DNA fragments that contain the desired sequences.

In another embodiment, an AC10 or HeFi-1 nucleic, most preferably an AC10 or HeFi-1 nucleic acid encoding the heavy or light chain variables region or a heavy or light chain CDR, can be chemically synthesized from the sequences disclosed herein. For example, the AC10 and HeFi-1 sequences can be synthesized by standard methods known in the art, *e.g.*, by use of an automated DNA synthesizer (such as those commercially available from Biosearch, Applied Biosystems, *etc.*). Alternatively, the AC10 or HeFi-1 nucleic acid may be synthesized by a commercially available service, for example by Blue Heron Biotechnology (Bothell, Washington) or QIAGEN Inc. (Valencia,

California). Other methods of isolating AC10 or HeFi-1 genes known to the skilled artisan can be employed.

The isolated AC10 or HeFi-1 nucleic acid (*e.g.*, a nucleic acid encoding AC10 or HeFi-1 or one or more CDRs or variable regions thereof) can then be inserted into an appropriate cloning vector. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, plasmids or modified viruses, but the vector system must be compatible with the host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as PBR322 or pUC plasmid derivatives or the Bluescript vector (Stratagene). The insertion into a cloning vector can, for example, be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. In an alternative method, the cleaved vector and an AC10 or HeFi-1 gene may be modified by homopolymeric tailing, or by PCR with primers containing the appropriate sequences. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, *etc.*, so that many copies of the gene sequence are generated. Antibodies comprising one or more CDRs from AC10 or HeFi-1 and framework regions from a different immunoglobulin molecule can be produced using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO 91/09967; U.S. Patent Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan, 1991, Molecular Immunology 28(4/5):489-498; Studnicka et al., 1994, Protein Engineering 7(6):805-814; Roguska et al., 1994, PNAS 91:969-973), and chain shuffling (U.S. Patent No. 5,565,332).

In a preferred embodiment of the present invention, an AC10 or HeFi-1 nucleic acid, for example a nucleic acid encoding an AC10 or HeFi-1 heavy or light chain variable region, can be cloned into an immunoglobulin expression vector. Briefly, the AC10 or HeFi-1 nucleic acid is synthesized or otherwise obtained by any of the methods

described herein, preferably flanked by appropriate restriction sites, then cloned into a vector suitable for expression of immunoglobulin molecules. A number of vectors have been described that contain, for example, sequences encoding immunoglobulin constant regions and restriction sites suitable for in frame cloning of antibody variable regions operably linked to a promoter and optionally a signal sequence useful for expression in a desired host cell. Non-limiting examples of vectors that have been designed for this purpose are described in McLean *et al.*, 2000, Mol Immunol. 37(14):837-45; Liang *et al.*, 2001, J. Immunol Methods 247(1-2):119-30; Persic *et al.*, 1997, Gene 187(1):9-18; Skerra, 1994, Gene 141(1):79-84; Walls *et al.*, 1993, Nucleic Acids Res 21(12):2921-29; Coloma *et al.*, 1992, J. Immunol. Methods 152(1):89-104. These vectors or similarly designed vectors can be used for cloning AC10 and HeFi-1 sequences for expression purposes.

In an alternative method, the desired gene may be identified and isolated after insertion into a suitable cloning vector in a "shot gun" approach. Enrichment for the desired gene, for example, by size fractionization, can be done before insertion into the cloning vector.

In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated AC10 or HeFi-1 gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

The AC10 or HeFi-1 sequences provided by the instant invention include those nucleotide sequences encoding substantially the same amino acid sequences as found in native AC10 or HeFi-1 variable regions, and those encoded amino acid sequences with functionally equivalent amino acids, as well as those encoding other AC10 or HeFi-1 derivatives or analogs, as described below for AC10 or HeFi-1 derivatives and analogs.

5.2 BINDING ASSAYS

As described above, the proteins, including antibodies, that are useful for the treatment of immunological disorders according to the methods of the present invention bind to CD30 and exert a cytostatic or cytotoxic effect on activated lymphocytes. Methods of demonstrating the ability of a protein to bind to CD30 are described herein.

Antibodies may be assayed for immunospecific binding to CD30 by any method known in the art. The immunoassays which can be used include but are not limited to competitive and non-competitive assay systems using techniques such as Western blots, radioimmunoassays, ELISA (enzyme linked immunosorbent assay), "sandwich" immunoassays, immunoprecipitation assays, precipitin reactions, gel diffusion precipitin reactions, immunodiffusion assays, agglutination assays, complement-fixation assays, immunoradiometric assays, fluorescent immunoassays, protein A immunoassays, to name but a few. Such assays are routine and well known in the art (*see, e.g.*, Ausubel *et. al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York, which is incorporated by reference herein in its entirety). Exemplary immunoassays are described briefly below (but are not intended by way of limitation).

Immunoprecipitation protocols generally comprise lysing a population of cells in a lysis buffer such as RIPA buffer (1% NP-40 or Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 0.01 M sodium phosphate at pH 7.2, 1% Trasylol) supplemented with protein phosphatase and/or protease inhibitors (*e.g.*, EDTA, PMSF, aprotinin, sodium vanadate), adding the antibody to the cell lysate, incubating for a period of time (*e.g.*, 1-4 hours) at 4° C, adding protein A and/or protein G sepharose beads to the cell lysate, incubating for about an hour or more at 4° C, washing the beads in lysis buffer and resuspending the beads in SDS/sample buffer. The ability of the antibody to immunoprecipitate CD30 can be assessed by, *e.g.*, Western blot analysis. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the binding of the antibody to CD30 and decrease the background (*e.g.*, pre-clearing the cell lysate with sepharose beads). For further discussion regarding

immunoprecipitation protocols *see, e.g.*, Ausubel *et al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.16.1.

Western blot analysis generally comprises preparing protein samples, electrophoresis of the protein samples in a polyacrylamide gel (*e.g.*, 8%- 20% SDS-PAGE depending on the molecular weight of the antigen), transferring the protein sample from the polyacrylamide gel to a membrane such as nitrocellulose, PVDF or nylon, incubating the membrane in blocking solution (*e.g.*, PBS with 3% BSA or non-fat milk), washing the membrane in washing buffer (*e.g.*, PBS-Tween 20), blotting the membrane with primary antibody (*i.e.*, the putative anti-CD30 antibody) diluted in blocking buffer, washing the membrane in washing buffer, incubating the membrane with a secondary antibody (which recognizes the primary antibody, *e.g.*, an anti-human antibody) conjugated to an enzyme substrate (*e.g.*, horseradish peroxidase or alkaline phosphatase) or radioactive molecule (*e.g.*, ^{32}P or ^{125}I) diluted in blocking buffer, washing the membrane in wash buffer, and detecting the presence of the secondary antibody. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected and to reduce the background noise. For further discussion regarding Western blot protocols *see, e.g.*, Ausubel *et al.*, eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 10.8.1.

ELISAs comprise preparing antigen (*i.e.*, CD30), coating the well of a 96 well microtiter plate with the CD30, adding the antibody conjugated to a detectable compound such as an enzyme (*e.g.*, horseradish peroxidase or alkaline phosphatase) to the well and incubating for a period of time, and detecting the presence of the antibody. In ELISAs the antibody does not have to be conjugated to a detectable compound; instead, a second antibody (which recognizes the antibody of interest) conjugated to a detectable compound may be added to the well. Further, instead of coating the well with the antigen, the antibody may be coated to the well. In this case, a second antibody conjugated to a detectable compound may be added following the addition of CD30 protein to the coated well. One of skill in the art would be knowledgeable as to the parameters that can be modified to increase the signal detected as well as other variations of ELISAs known in the art. For further discussion regarding ELISAs *see, e.g.*, Ausubel

et al., eds., 1994, Current Protocols in Molecular Biology, Vol. 1, John Wiley & Sons, Inc., New York at 11.2.1.

The binding affinity of an antibody to CD30 and the off-rate of an antibody CD30 interaction can be determined by competitive binding assays. One example of a competitive binding assay is a radioimmunoassay comprising the incubation of labeled CD30 (*e.g.*, ^{3}H or ^{125}I) with the antibody of interest in the presence of increasing amounts of unlabeled CD30, and the detection of the antibody bound to the labeled CD30. The affinity of the antibody for CD30 and the binding off-rates can then be determined from the data by Scatchard plot analysis. Competition with a second antibody (such as AC10 or HeFi-1) can also be determined using radioimmunoassays. In this case, CD30 is incubated with the antibody of interest conjugated to a labeled compound (*e.g.*, ^{3}H or ^{125}I) in the presence of increasing amounts of an unlabeled second antibody. Alternatively, the binding affinity of an antibody to CD30 and the on- and off-rates of an antibody-CD30 interaction can be determined by surface plasmon resonance.

Proteins that are useful in the methods of the invention may also be assayed for their ability to bind to CD30 by a standard assay known in the art. Such assays include far Westerns and the yeast two hybrid system. These assays are described in Section 5.2, *supra*. Another variation on the far Western technique described above entails measuring the ability of a labeled candidate protein to bind to CD30 in a Western blot. In one non-limiting example of a far Western blot, CD30 or the fragment thereof of interest is expressed as a fusion protein further comprising glutathione-S-transferase (GST) and a protein serine/threonine kinase recognition site (such as a cAMP-dependent kinase recognition site). The fusion protein is purified on glutathione-Sepharose beads (Pharmacia Biotech) and labeled with bovine heart kinase (Sigma) and 100 μCi of ^{32}P-ATP (Amersham). The test protein(s) of interest are separated by SDS-PAGE and blotted to a nitrocellulose membrane, then incubated with the labeled CD30. Thereafter, the membrane is washed and the radioactivity quantitated. Conversely, the protein of interest can be labeled by the same method and used to probe a nitrocellulose membrane onto which CD30 has been blotted.

5.3 SEQUENCES RELATED TO AC10 AND HeFi-1

The present invention further encompasses the use of proteins and nucleic acids comprising a region of homology to CDRs of AC10 and HeFi-1, or the coding regions therefor, respectively, for the treatment or prevention of an immunological disorder. In various embodiments, the region of homology is characterized by at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity with the corresponding region of AC10 or HeFi-1.

In one embodiment, the present invention provides a method of treating or preventing an immunological disorder comprising administering to a patient in need thereof a protein with a region of homology to a CDR of HeFi-1 (SEQ ID NO:20, SEQ ID NO:22; SEQ ID NO:24; SEQ ID NO:28, SEQ ID NO:30 or SEQ ID NO:32), provided that the protein induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes. In another embodiment, the present invention provides a method of treating or preventing an immunological disorder comprising administering to a patient in need thereof a protein with a region of homology to a CDR of AC10 (SEQ ID NO:4; SEQ ID NO:6; SEQ ID NO:8; SEQ ID NO:12; SEQ ID NO:14; or SEQ ID NO:16), provided that the protein induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

In another embodiment, the present invention provides a method of treating or preventing an immunological disorder comprising administering to a patient in need thereof a nucleic acid with a region of homology to a CDR coding region of HeFi-1 (SEQ ID NO:19, SEQ ID NO:21, SEQ ID NO:23, SEQ ID NO:27, SEQ ID NO:29 or SEQ ID NO:31), provided that the encoded protein induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes. In yet another embodiment, the present invention provides a method of treating or preventing an immunological disorder comprising administering to a patient in need thereof a nucleic acid with a region of homology to a CDR coding region of AC10 (SEQ ID NO:3, SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:11, SEQ ID NO:13, SEQ ID NO:15), provided that the

encoded protein induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes.

The present invention further encompasses methods of treating or preventing an immunological disorder comprising administering to a patient in need thereof a protein or nucleic acids comprising a region of homology to the variable regions of AC10 and HeFi-1, or the coding region therefor, respectively. In various embodiments, the region of homology is characterized by at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or at least 98% identity with the corresponding region of AC10 or HeFi-1.

In one embodiment, the present invention provides methods of treating or preventing an immunological disorder comprising administering to a patient in need thereof a protein with a region of homology to a variable region of HeFi-1 (SEQ ID NO:18 or SEQ ID NO: 26). In another embodiment, the present invention provides methods of treating or preventing an immunological disorder comprising administering to a patient in need thereof a protein with a region of homology to a variable region of AC10 (SEQ ID NO: 2 or SEQ ID NO: 10).

In one embodiment, the present invention provides methods of treating or preventing an immunological disorder comprising administering to a patient in need thereof a nucleic acid with a region of homology to a variable region coding region of HeFi-1 (SEQ ID NO:17 or SEQ ID NO:25). In another embodiment, the present invention provides methods of treating or preventing an immunological disorder comprising administering to a patient in need thereof a nucleic with a region of homology to a variable region coding region of AC10 (SEQ ID NO:1 or SEQ ID NO:9).

To determine the percent identity of two amino acid sequences or of two nucleic acids, *e.g.* between the sequences of an AC10 or HeFi-1 variable region and sequences from other proteins with regions of homology to the AC10 or HeFi-1 variable region, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then

compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % identity = # of identical positions/total # of positions (*e.g.*, overlapping positions) x 100). In one embodiment, the two sequences are the same length.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul, 1990, Proc. Natl. Acad. Sci. USA 87:2264-2268, modified as in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, *et al.*, 1990, J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to a nucleic acid encoding a SCA-1 modifier protein. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to a SCA-1 modifier protein. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, 1997, Nucleic Acids Res. 25:3389-3402. Alternatively, PSI-Blast can be used to perform an iterated search which detects distant relationships between molecules (*Id.*). When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (*e.g.*, XBLAST and NBLAST) can be used. *See* http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Additional algorithms for sequence analysis are known in the art and include ADVANCE and ADAM as described in Torellis and Robotti, 1994, Comput. Appl. Biosci., 10:3-5; and FASTA described in Pearson and Lipman, 1988, Proc. Natl. Acad. Sci. 85:2444-8. Within FASTA, ktup is a

control option that sets the sensitivity and speed of the search. If ktup=2, similar regions in the two sequences being compared are found by looking at pairs of aligned residues; if ktup=1, single aligned amino acids are examined. ktup can be set to 2 or 1 for protein sequences, or from 1 to 6 for DNA sequences. The default if ktup is not specified is 2 for proteins and 6 for DNA. For a further description of FASTA parameters, see http://bioweb.pasteur.fr/docs/man/man/fasta.1.html#sect2, the contents of which are incorporated herein by reference.

Alternatively, protein sequence alignment may be carried out using the CLUSTAL W algorithm, as described by Higgins *et al.*, 1996, Methods Enzymol. 266:383-402.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

5.4 METHODS OF PRODUCING THE PROTEINS OF THE INVENTION

The proteins, including antibodies, that are useful in the methods of the present invention can be produced by any method known in the art for the synthesis of proteins, in particular, by chemical synthesis or preferably, by recombinant expression techniques.

Recombinant expression of a protein that binds to CD30 and induces CD30 signaling in a lymphocyte and/or exerts a cytotoxic or cytostatic effect on activated lymphocytes requires construction of an expression vector containing a nucleic acid that encodes the protein. Once a nucleic acid encoding such a protein has been obtained, the vector for the production of the protein molecule may be produced by recombinant DNA technology using techniques well known in the art. Thus, methods for preparing a protein by expressing a nucleic acid containing nucleotide sequence encoding said protein are described herein. Methods which are well known to those skilled in the art can be used to construct expression vectors containing coding sequences and appropriate transcriptional and translational control signals. These methods include, for example, *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* genetic recombination.

The invention, thus, provides replicable vectors comprising a nucleotide sequence encoding a protein of the invention operably linked to a promoter. Wherein the protein is an antibody, the nucleotide sequence may encode a heavy or light chain thereof, or a heavy or light chain variable domain, operably linked to a promoter. Such vectors may include the nucleotide sequence encoding the constant region of the antibody molecule (*see, e.g.*, PCT Publication WO 86/05807; PCT Publication WO 89/01036; and U.S. Patent No. 5,122,464) and the variable domain of the antibody may be cloned into such a vector for expression of the entire heavy or light chain.

The expression vector is transferred to a host cell by conventional techniques and the transfected cells are then cultured by conventional techniques to produce a protein of the invention. Thus, the invention encompasses host cells containing a nucleic acid encoding a protein of the invention, operably linked to a heterologous promoter. In preferred embodiments for the expression of double-chained antibodies, vectors encoding both the heavy and light chains may be co-expressed in the host cell for expression of the entire immunoglobulin molecule, as detailed below.

A variety of host-expression vector systems may be utilized to express the proteins molecules of the invention. Such host-expression systems represent vehicles by which the coding sequences of interest may be produced and subsequently purified, but also represent cells which may, when transformed or transfected with the appropriate nucleotide coding sequences, express a protein of the invention *in situ*. These include but are not limited to microorganisms such as bacteria (*e.g.*, *E. coli*, *B. subtilis*) transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing antibody coding sequences; yeast (*e.g.*, Saccharomyces, Pichia) transformed with recombinant yeast expression vectors containing antibody coding sequences; insect cell systems infected with recombinant virus expression vectors (*e.g.*, baculovirus) containing antibody coding sequences; plant cell systems infected with recombinant virus expression vectors (*e.g.*, cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors (*e.g.*, Ti plasmid) containing antibody coding sequences; or mammalian cell systems (*e.g.*, COS, CHO, BHK, 293, 3T3 cells) harboring recombinant expression constructs containing promoters derived from the genome of mammalian cells (*e.g.*, metallothionein promoter) or from mammalian viruses

(*e.g.*, the adenovirus late promoter; the vaccinia virus 7.5K promoter). Preferably, bacterial cells such as *Escherichia coli*, and more preferably, eukaryotic cells, especially for the expression of whole recombinant antibody molecules, are used for the expression of a recombinant protein of the invention. For example, mammalian cells such as Chinese hamster ovary cells (CHO), in conjunction with a vector such as the major intermediate early gene promoter element from human cytomegalovirus is an effective expression system for proteins of the invention (Foecking *et al.*, 1986, Gene 45:101; Cockett *et al.*, 1990, Bio/Technology 8:2).

In bacterial systems, a number of expression vectors may be advantageously selected depending upon the use intended for the folding and post-translation modification requirements protein being expressed. Where possible, when a large quantity of such a protein is to be produced, for the generation of pharmaceutical compositions comprising a protein of the invention, vectors which direct the expression of high levels of fusion protein products that are readily purified may be desirable. Such vectors include, but are not limited, to the *E. coli* expression vector pUR278 (Ruther *et al.*, 1983, EMBO 1. 2:1791), in which the antibody coding sequence may be ligated individually into the vector in frame with the lac Z coding region so that a fusion protein is produced; pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res. 13:3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem. 24:5503-5509); and the like. pGEX vectors may also be used to express fusion proteins with glutathione S-transferase (GST). In general, such fusion proteins are soluble and can easily be purified from lysed cells by adsorption and binding to matrix glutathioneagarose beads followed by elution in the presence of free glutathione. The pGEX vectors are designed to include thrombin or factor Xa protease cleavage sites so that the cloned target gene product can be released from the GST moiety.

In an insect system, *Autographa californica* nuclear polyhedrosis virus (AcNPV) is used as a vector to express foreign genes. The virus grows in *Spodoptera frugiperda* cells. The antibody coding sequence may be cloned individually into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin promoter).

In mammalian host cells, a number of viral-based expression systems may be utilized. In cases where an adenovirus is used as an expression vector, the coding

sequence of the protein of the invention may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non- essential region of the viral genome (*e.g.*, region El or E3) will result in a recombinant virus that is viable and capable of expressing the protein of the invention in infected hosts. (*See, e.g.*, Logan & Shenk, 1984, Proc. Natl. Acad. Sci. USA 8 1:355-359). Specific initiation signals may also be required for efficient translation of inserted coding sequences. These signals include the ATG initiation codon and adjacent sequences. Furthermore, the initiation codon must be in phase with the reading frame of the desired coding sequence to ensure translation of the entire insert. These exogenous translational control signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see Bittner *et al.*, 1987, Methods in Enzymol. 153:51-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Such modifications (*e.g.*, glycosylation) and processing (*e.g.*, cleavage) of protein products may be important for the function of the protein of the invention. Different host cells have characteristic and specific mechanisms for the post-translational processing and modification of proteins and gene products. Appropriate cell lines or host systems can be chosen to ensure the correct modification and processing of the foreign protein expressed. To this end, eukaryotic host cells which possess the cellular machinery for proper processing of the primary transcript, glycosylation, and phosphorylation of the gene product may be used. Such mammalian host cells include but are not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293, 3T3, and W138.

For long-term, high-yield production of recombinant proteins that bind to CD30 and exert a cytotoxic or cytostatic effect on activated lymphocytes, stable expression is preferred. For example, cell lines which stably express the protein may be engineered. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with DNA controlled by appropriate expression control elements (*e.g.*, promoter, enhancer, sequences, transcription terminators,

polyadenylation sites, etc.), and a selectable marker. Following the introduction of the foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows cells to stably integrate the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the protein of the invention.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler *et al.*, 1977, Cell 11:223), hypoxanthineguanine phosphoribosyltransferase (Szybalska & Szybalski, 1992, Proc. Natl. Acad. Sci. USA 48:202), and adenine phosphoribosyltransferase (Lowy *et al.*, 1980, Cell 22:8-17) genes can be employed in tk-, hgprt- or aprt- cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for the following genes: dhfr, which confers resistance to methotrexate (Wigler *et al.*, 1980, Proc. Natl. Acad. Sci. USA 77:357; O'Hare *et al.*, 1981, Proc. Natl. Acad. Sci. USA 78:1527); gpt, which confers resistance to mycophenolic acid (Mulligan & Berg, 1981, Proc. Natl. Acad. Sci. USA 78:2072); neo, which confers resistance to the aminoglycoside G-418 (Goldspiel *et al.*, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95 ; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932 ; and Morgan and Anderson, 1993, Ann. Rev. Biochem. 62: 191-217; May, 1993, TIB TECH 11(5):155-215); and hygro, which confers resistance to hygromycin (Santerre *et al.*, 1984, Gene 30:147). Methods commonly known in the art of recombinant DNA technology may be routinely applied to select the desired recombinant clone, and such methods are described, for example, in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990); and in Chapters 12 and 13, Dracopoli *et al.* (eds), Current Protocols in Human Genetics, John Wiley & Sons, NY (1994); Colberre-Garapin *et al.*, 1981, J. Mol. Biol. 150:1, which are incorporated by reference herein in their entireties.

The expression levels of a protein of the invention can be increased by vector amplification (for a review, see Bebbington and Hentschel, "The Use of Vectors

Based on Gene Amplification for the Expression of Cloned Genes in Mammalian Cells in DNA Cloning", Vol.3. (Academic Press, New York, 1987)). When a marker in the vector system expressing antibody is amplifiable, increase in the level of inhibitor present in culture of host cell will increase the number of copies of the marker gene. Since the amplified region is associated with the antibody gene, production of the protein of the invention will also increase (Crouse *et al.*, 1983, Mol. Cell. Biol. 3:257).

Wherein the protein of the invention is an antibody, the host cell may be co-transfected with two expression vectors of the invention, the first vector encoding a heavy chain derived protein and the second vector encoding a light chain derived protein. The two vectors may contain identical selectable markers which enable equal expression of heavy and light chain proteins. Alternatively, a single vector may be used which encodes, and is capable of expressing, both heavy and light chain proteins. In such situations, the light chain should be placed before the heavy chain to avoid an excess of toxic free heavy chain (Proudfoot, 1986, Nature 322:52; Kohler, 1980, Proc. Natl. Acad. Sci. USA 77:2197). The coding sequences for the heavy and light chains may comprise cDNA or genomic DNA.

Once a protein molecule of the invention has been produced by an animal, chemically synthesized, or recombinantly expressed, it may be purified by any method known in the art for purification of proteins, for example, by chromatography (*e.g.*, ion exchange; affinity, particularly by affinity for the specific antigen, Protein A (for antibody molecules, or affinity for a heterologous fusion partner wherein the protein is a fusion protein; and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

The present invention encompasses the use of proteins of the invention that are fusion proteins, *i.e.*, proteins that are recombinantly fused or chemically conjugated (including both covalent and non-covalent conjugation) to heterologous proteins (of preferably at least 10, 20, 30, 40, 50, 60, 70, 80, 90 or at least 100 amino acids). The fusion does not necessarily need to be direct, but may occur through linker sequences.

The present invention further includes compositions comprising proteins of the invention fused or conjugated to antibody domains other than the variable regions.

For example, the proteins of the invention may be fused or conjugated to an antibody Fc region, or portion thereof. The antibody portion fused to a protein of the invention may comprise the constant region, hinge region, CH 1 domain, CR2 domain, and CH3 domain or any combination of whole domains or portions thereof. The proteins may also be fused or conjugated to the above antibody portions to form multimers. For example, Fc portions fused to the proteins of the invention can form dimers through disulfide bonding between the Fc portions. Higher multimeric forms can be made by fusing the proteins to portions of IgA and IgM. Methods for fusing or conjugating the proteins of the invention to antibody portions are known in the art. *See, e.g.*, U.S. Patent Nos. 5,336,603; 5,622,929; 5,359,046; 5,349,053; 5,447,851; 5,112,946; EP 307,434; EP 367,166; PCT publications WO 96/04388; WO 9 1/06570; Ashkenazi *et al.*, 1991, Proc. Nat. Acad. Sci. USA 88:10535-10539; Zheng *et al.*, 1995, J. Immunol. 154:5590-5600; and Vil *et al.*, 1992, Proc. Natl. Acad. Sci. USA 89:11337- 11341 (said references incorporated by reference in their entireties).

5.5 CONJUGATES AND FUSION PROTEINS

As discussed, *supra*, the methods of the invention for treatment and prevention of immunological disorders encompass the use of proteins that bind to CD30 and exert a cytostatic and/or cytotoxic effect on activated lymphocytes, and that are further fused or conjugated to heterologous proteins or cytotoxic agents.

The present invention thus provides for methods of treatment or prevention of immunological disorders by administration of a protein or nucleic acid of the invention. In certain embodiments of the invention, a protein or nucleic acid of the invention may be chemically modified to improve its cytotoxic and/or cytostatic properties. For example, a protein of the invention can be administered as a conjugate. Particularly suitable moieties for conjugation to proteins of the invention are chemotherapeutic agents, pro-drug converting enzymes, radioactive isotopes or compounds, or toxins.

In one embodiment, a protein of the invention is fused to a marker sequence, such as a peptide, to facilitate purification. In preferred embodiments, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE

vector (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311), among others, many of which are commercially available. As described in Gentz *et al.*, 1989, Proc. Natl. Acad. Sci. USA 86:821-824, for instance, hexa-histidine provides for convenient purification of the fusion protein. Other peptide tags useful for purification include, but are not limited to, the "HA" tag, which corresponds to an epitope derived from the influenza hemagglutinin protein (Wilson *et al.*, 1984, Cell 37:767) and the "flag" tag. Such fusion proteins can be generated by standard recombinant methods known to those of skill in the art.

In another embodiment, the proteins of the invention are fused or conjugated to a therapeutic agent. For example, a protein of the invention may be conjugated to a cytotoxic agent such as a chemotherapeutic agent (see *infra* Section 5.6), a toxin (*e.g.*, a cytostatic or cytocidal agent), or a radionuclide (*e.g.*, alpha-emitters such as, for example, ^{212}Bi, ^{211}At, or beta-emitters such as, for example, ^{131}I, ^{90}Y, or ^{67}Cu). Examples of additional agents that are useful for conjugating to the anti-CD30 molecules of the invention are provided in Section 5.12.1, *infra*.

The conjugates of the invention used for enhancing the therapeutic effect of the anti-CD30 antibodies that are useful in the methods of the present invention include non-classical therapeutic agents such as toxins. Such toxins include, but are not limited to, abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin.

Alternatively, an antibody of the invention can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980, which is incorporated herein by reference in its entirety. Heteroconjugates useful for practicing the present invention comprise antibodies or antibody portions that bind to CD30 (including but not limited to antibodies that have the CDRs and/or heavy chains of the monoclonal antibodies Ki-2, Ki-4, Ki-5, Ki-7, Ber-H2, HRS-1, HRS-4, Ki-1, Ki-6, M67, Ki-3, M44, HeFi-1, and AC10) and antibody or a antibody portions that bind to a lymphocyte surface receptor or receptor complex, such as an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin (C-type, S-type, or I-type), or a complement control protein. Examples of such molecules, and

antibodies against such molecules that can be used to make heteroconjugates, are provided in Section 5.12.2, *infra*.

As discussed above, in certain embodiments of the invention, a protein of the invention can be co-administered with a pro-drug converting enzyme. The pro-drug converting enzyme can be expressed as a fusion protein with or conjugated to a protein of the invention. Exemplary pro-drug converting enzymes are carboxypeptidase G2, beta-glucuronidase, penicillin-V-amidase, penicillin-G-amidase, β-lactamase, β-glucosidase, nitroreductase and carboxypeptidase A.

5.6 ANTIBODY-DRUG CONJUGATES

The present invention encompasses the use of anti-CD30 antibody-drug conjugates (anti-CD30 ADCs) for the treatment or prevention of an immunological disorder. The ADCs of the invention are tailored to produce clinically beneficial cytotoxic or cytostatic effects on CD30-expressing cells when administered to a patient with an immune disorder involving CD30-expressing cells, preferably when administered alone but also in combination with other therapeutic agents.

Techniques for conjugating such drugs to proteins, and in particular to antibodies, are well known, *see, e.g.*, Arnon *et al.*, "Monoclonal Antibodies For Immunotargeting Of Drugs In Cancer Therapy", in Monoclonal Antibodies And Cancer Therapy, Reisfeld *et al.* (eds.), pp. 243-56 (Alan R. Liss, Inc., 1985); Hellstrom *et al.*, "Antibodies For Drug Delivery", in Controlled Drug Delivery (2nd ed.), Robinson *et al.* (eds.), pp. 623-53 (Marcel Dekker, Inc., 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In Cancer Therapy: A Review", in Monoclonal Antibodies '84: Biological And Clinical Applications, Pinchera *et al.* (eds.), pp. 475-506 (1985); "Analysis, Results, And Future Prospective Of The Therapeutic Use Of Radiolabeled Antibody In Cancer Therapy", in Monoclonal Antibodies For Cancer Detection And Therapy, Baldwin *et al.* (eds.), pp. 303-16 (Academic Press 1985), and Thorpe *et al.*, 1982, Immunol. Rev. 62:119-58.

Because in many of the disease states that are encompassed by the treatment methods of the present invention a significant amount of soluble CD30 is shed

from the activated lymphocytes, it is preferable when using an anti-CD30 antibody that is conjugated to a drug (*e.g.*, a cytotoxic agent or an immunosuppressive agent) or prodrug converting enzyme that the drug or prodrug converting enzyme is active in the vicinity of the activated lymphocytes rather than any place in the body that soluble CD30 may be found.

Two approaches may be taken to minimize drug activity outside the activated lymphocytes that are targeted by the anti-CD30 antibodies of the invention: first, an antibody that binds to cell membrane but not soluble CD30 may be used, so that the drug, including drug produced by the actions of the prodrug converting enzyme, is concentrated at the cell surface of the activated lymphocyte. A more preferred approach for minimizing the activity of drugs bound to the antibodies of the invention is to conjugate the drugs in a manner that would reduce their activity unless they are hydrolyzed or cleaved off the antibody. Such methods would employ attaching the drug to the antibodies with linkers that are sensitive to the environment at the cell surface of the activated lymphocyte (*e.g.*, the activity of a protease that is present at the cell surface of the activated lymphocyte) or to the environment inside the activated lymphocyte the conjugate encounters when it is taken up by the activated lymphocyte (*e.g.*, in the endosomal or, for example by virtue of pH sensitivity or protease sensitivity, in the lysosomal environment).

In one embodiment, the linker is an acid- labile hydrazone or hydrazide group that is hydrolyzed in the lysosome (*see, e.g.*, U.S. Patent No. 5,622,929) In alternative embodiments, drugs can be appended to anti-CD30 antibodies through other acid-labile linkers, such as cis-aconitic amides, orthoesters, acetals and ketals (Dubowchik and Walker, 1999, Pharm. Therapeutics 83:67-123; Neville *et al.*, 1989, Biol. Chem. 264:14653-14661). Such linkers are relatively stable under neutral pH conditions, such as those in the blood, but are unstable at below pH 5, the approximate pH of the lysosome.

In other embodiments, drugs are attached to the anti-CD30 antibodies of the invention using peptide spacers that are cleaved by intracellular proteases. Target enzymes include cathepsins B and D and plasmin, all of which are known to hydrolyze dipeptide drug derivatives resulting in the release of active drug inside target cells

(Dubowchik and Walker, 1999, Pharm. Therapeutics 83:67-123). The advantage of using intracellular proteolytic drug release is that the drug is highly attenuated when conjugated and the serum stabilities of the conjugates can be extraordinarily high.

In yet other embodiments, the linker is a malonate linker (Johnson *et al.*, 1995, Anticancer Res. 15:1387-93), a maleimidobenzoyl linker (Lau *et al.*, 1995, Bioorg-Med-Chem. 3(10):1299-1304), or a 3'-N-amide analog (Lau *et al.*, 1995, Bioorg-Med-Chem. 3(10):1305-12).

The drugs used for conjugation to the anti-CD30 antibodies of the present invention can include conventional chemotherapeutics, such as doxorubicin, paclitaxel, melphalan, vinca alkaloids, methotrexate, mitomycin C, etoposide, and others. In addition, potent agents such CC-1065 analogues, calichiamicin, maytansine, analogues of dolastatin 10, rhizoxin, and palytoxin can be linked to the anti-CD30 antibodies using the conditionally stable linkers to form potent immunoconjugates. Examples of other suitable drugs for conjugation to the anti-CD30 antibodies of the present invention are provided in Section 5.12.1, *infra*.

5.6.1 LINKERS

As discussed above in Section 5.6, ADCs are generally made by conjugating a drug to an antibody through a linker. Thus, a majority of the ADCs of the present invention, which comprise an anti-CD30 antibody and a high potency drug and/or an internalization-promoting drug, further comprise a linker. Any linker that is known in the art may be used in the ADCs of the present invention, *e.g.*, bifunctional agents (such as dialdehydes or imidoesters) or branched hydrazone linkers (*see*, *e.g.*, U.S. Patent No. 5,824,805, which is incorporated by reference herein in its entirety).

In certain, non-limiting, embodiments of the invention, the linker region between the drug moiety and the antibody moiety of the anti-CD30 ADC is cleavable or hydrolyzable under certain conditions, wherein cleavage or hydrolysis of the linker releases the drug moiety from the antibody moiety. Preferably, the linker is sensitive to cleavage or hydrolysis under intracellular conditions.

In a preferred embodiment, the linker region between the drug moiety and the antibody moiety of the anti-CD30 ADC is hydrolyzable if the pH changes by a certain

value or exceeds a certain value. In a particularly preferred embodiment of the invention, the linker is hydrolyzable in the milieu of the lysosome, *e.g.*, under acidic conditions (*i.e.*, a pH of around 5-5.5 or less). In other embodiments, the linker is a peptidyl linker that is cleaved by a peptidase or protease enzyme, including but not limited to a lysosomal protease enzyme, a membrane-associated protease, an intracellular protease, or an endosomal protease. Preferably, the linker is at least two amino acids long, more preferably at least three amino acids long. Peptidyl linkers that are cleavable by enzymes that are present in CD30-expressing cancers are preferred. For example, a peptidyl linker that is cleavable by cathepsin-B (*e.g.*, a Gly-Phe-Leu-Gly linker), a thiol-dependent protease that is highly expressed in cancerous tissue, can be used. Other such linkers are described, *e.g.*, in U.S. Patent No. 6,214,345, which is incorporated by reference in its entirety herein.

In other, non-mutually exclusive embodiments of the invention, the linker by which the anti-CD30 antibody and the drug of an ADC of the invention are conjugated promotes cellular internalization. In certain embodiments, the linker-drug moiety of the ADC promotes cellular internalization. In certain embodiments, the linker is chosen such that the structure of the entire ADC promotes cellular internalization.

In a specific embodiment of the invention, derivatives of valine-citrulline are used as linker (val-cit linker). The synthesis of doxorubicin with the val-cit linker have been previously described (U.S. patent 6,214,345 to Dubowchik and Firestone, which is incorporated by reference herein in its entirety).

In another specific embodiment, the linker is a phe-lys linker.

In another specific embodiment, the linker is a thioether linker (*see*, *e.g.*, U.S. Patent No. 5,622,929 to Willner *et al.*, which is incorporated by reference herein in its entirety).

In yet another specific embodiment, the linker is a hydrazone linker (*see, e.g.*, U.S. Patent Nos. 5,122,368 to Greenfield *et al.* and 5,824,805 to King *et al.*, which are incorporated by reference herein in their entireties).

In yet other specific embodiments, the linker is a disulfide linker. A variety of disulfide linkers are known in the art, including but not limited to those that can be formed using SATA (N-succinimidyl-S-acetylthioacetate), SPDP (N-succinimidyl-3-

(2-pyridyldithio)propionate), SPDB (N-succinimidyl-3-(2-pyridyldithio)butyrate) and SMPT (N-succinimidyl-oxycarbonyl-alpha-methyl-alpha-(2-pyridyl-dithio)toluene). SPDB and SMPT (see, e.g., Thorpe *et al.*, 1987, Cancer Res., 47:5924-5931; Wawrzynczak *et al.*,1987, In Immunoconjugates: Antibody Conjugates in Radioimagery and Therapy of Cancer, ed. C. W. Vogel, Oxford U. Press, pp. 28-55; *see also* U.S. Patent No. 4,880,935 to Thorpe *et al.*, which is incorporated by reference herein in its entirety).

A variety of linkers that can be used with the compositions and methods of the present invention are described in U.S. provisional application no. 60/400,403, entitled "Drug Conjugates and their use for treating cancer, an autoimmune disease or an infectious disease", by Inventors: Peter D. Senter, Svetlana Doronina and Brian E. Toki, submitted on July 31, 2002, which is incorporated by reference in its entirety herein.

In yet other embodiments of the present invention, the linker unit of an anti-CD30 antibody-linker-drug conjugate (anti-CD30 ADC) links the cytotoxic or cytostatic agent (drug unit; -D) and the anti-CD30 antibody unit (-A). As used herein the term anti-CD30 ADC encompasses anti-CD30 antibody drug conjugates with and without a linker unit. The linker unit has the general formula:

$$—T_a—W_w—Y_y—$$

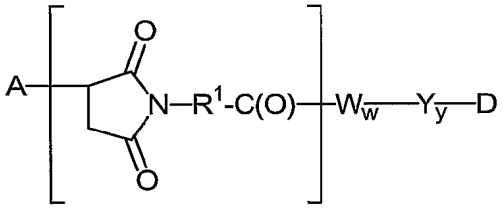
wherein:
-T- is a stretcher unit;
a is 0 or 1;
each -W- is independently an amino acid unit;
w is independently an integer ranging from 2 to12;
-Y- is a spacer unit; and
y is 0, 1 or 2.

5.6.1.1 THE STRETCHER UNIT

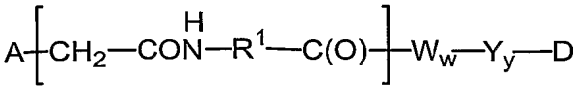
The stretcher unit (-T-), when present, links the anti-CD30 antibody unit to an amino acid unit (-W-). Useful functional groups that can be present on an anti-CD30 antibody, either naturally or via chemical manipulation include, but are not limited to, sulfhydryl, amino, hydroxyl, the anomeric hydroxyl group of a carbohydrate, and carboxyl. Preferred functional groups are sulfhydryl and amino. Sulfhydryl groups can be generated by reduction of the intramolecular disulfide bonds of an anti-CD30 antibody. Alternatively, sulfhydryl groups can be generated by reaction of an amino group of a

lysine moiety of an anti-CD30 antibody with 2-iminothiolane (Traut's reagent) or other sulfhydryl generating reagents. In specific embodiments, the anti-CD30 antibody is a recombinant antibody and is engineered to carry one or more lysines. In other embodiments, the recombinant anti-CD30 antibody is engineered to carry additional sulfhydryl groups, *e.g.*, additional cysteines.

In certain specific embodiments, the stretcher unit forms a bond with a sulfur atom of the anti-CD30 antibody unit. The sulfur atom can be derived from a sulfhydryl (-SH) group of a reduced anti-CD30 antibody (A). Representative stretcher units of these embodiments are depicted within the square brackets of Formulas (**Ia**) and (**Ib**; see *infra*), wherein A-, -W-, -Y-, -D, w and y are as defined above and R^1 is selected from -C_1-C_{10} alkylene-, -C_3-C_8 carbocyclo-, -O-(C_1-C_8alkyl)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -arylene-C_1-C_{10} alkylene-, -C_1-C_{10} alkylene-(C_3-C_8 carbocyclo)-, -(C_3-C_8 carbocyclo)-C_1-C_{10} alkylene-, -C_3-C_8 heterocyclo-, -C_1-C_{10} alkylene-(C_3-C_8 heterocyclo)-, -(C_3-C_8 heterocyclo)-C_1-C_{10} alkylene-, -$(CH_2CH_2O)_r$-, and -$(CH_2CH_2O)_r$-CH_2-; and r is an integer ranging from 1-10.

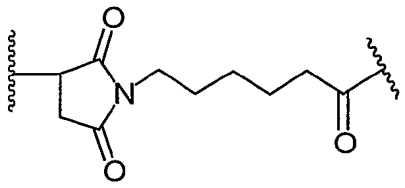


(Ia)

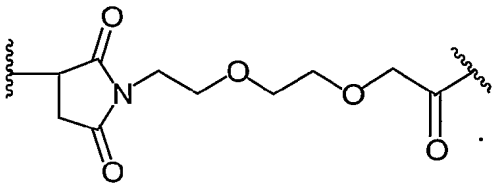


(Ib)

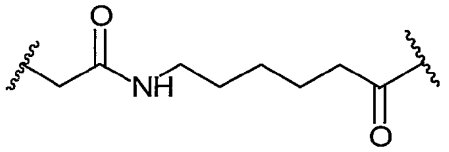
An illustrative stretcher unit is that of formula (**Ia**) where R^1 is -$(CH_2)_5$-:



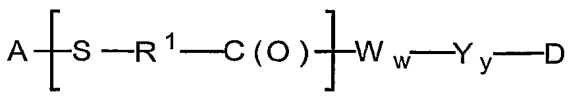
Another illustrative stretcher unit is that of formula (**Ia**) where R^1 is $-(CH_2CH_2O)_r-CH_2-$; and r is 2:



Still another illustrative stretcher unit is that of formula (**Ib**) where R^1 is $-(CH_2)_5-$:

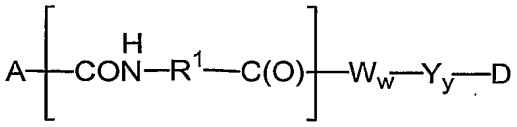


In certain other specific embodiments, the stretcher unit is linked to the anti-CD30 antibody unit (A) via a disulfide bond between a sulfur atom of the anti-CD30 antibody unit and a sulfur atom of the stretcher unit. A representative stretcher unit of this embodiment is depicted within the square brackets of Formula (**II**), wherein R^1, A-, -W-, -Y-, -D, w and y are as defined above.

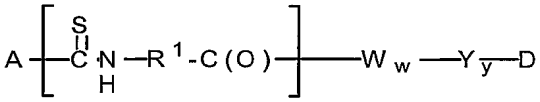


(**II**)

In even other specific embodiments, the reactive group of the stretcher contains a reactive site that can be reactive to an amino group of an anti-CD30 antibody. The amino group can be that of an arginine or a lysine. Suitable amine reactive sites include, but are not limited to, activated esters such as succinimide esters, 4-nitrophenyl esters, pentafluorophenyl esters, anhydrides, acid chlorides, sulfonyl chlorides, isocyanates and isothiocyanates. Representative stretcher units of these embodiments are depicted within the square brackets of Formulas (**IIIa**) and (**IIIb**), wherein R^1, A-, -W-, -Y-, -D, w and y are as defined above;



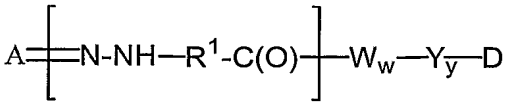
(IIIa)



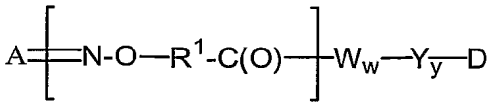
(IIIb)

In yet another aspect of the invention, the reactive function of the stretcher contains a reactive site that is reactive to a modified carbohydrate group that can be present on an anti-CD30 antibody. In a specific embodiment, the anti-CD30 antibody is glycosylated enzymatically to provide a carbohydrate moiety. The carbohydrate may be mildly oxidized with a reagent such as sodium periodate and the resulting carbonyl unit of the oxidized carbohydrate can be condensed with a stretcher that contains a functionality such as a hydrazide, an oxime, a reactive amine, a hydrazine, a thiosemicarbazone, a hydrazine carboxylate, and an arylhydrazide such as those described by Kaneko, T. *et al.* *Bioconjugate Chem* **1991**, 2, 133-41. Representative stretcher units of this embodiment

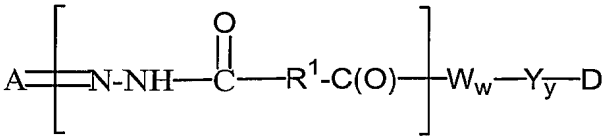
are depicted within the square brackets of Formulas (**IVa**)-(**IVc**), wherein R^1, A-, -W-, -Y-, -D, w and y are as defined above.



(IVa)



(IVb)

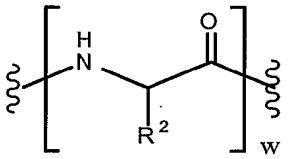


(IVc)

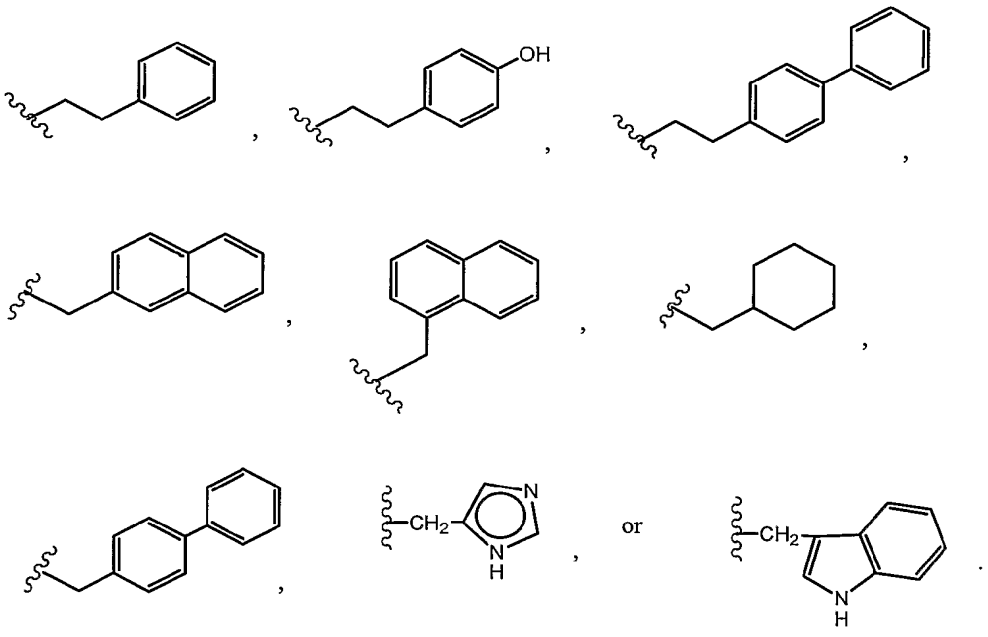
5.6.1.2 THE AMINO ACID UNIT

The amino acid unit (-W-) links the stretcher unit (-T-) to the Spacer unit (-Y-) if the Spacer unit is present, and links the stretcher unit to the cytotoxic or cytostatic agent (Drug unit; D) if the spacer unit is absent.

- W_w- is a dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide, decapeptide, undecapeptide or dodecapeptide unit. Each -W- unit independently has the formula denoted below in the square brackets, and w is an integer ranging from 2 to 12:

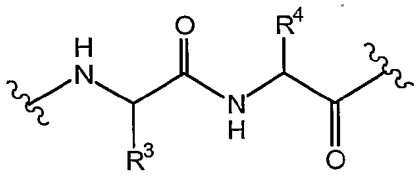


wherein R^2 is hydrogen, methyl, isopropyl, isobutyl, *sec*-butyl, benzyl, *p*-hydroxybenzyl, -CH_2OH, -$CH(OH)CH_3$, -$CH_2CH_2SCH_3$, -CH_2CONH_2, -CH_2COOH, -$CH_2CH_2CONH_2$, -CH_2CH_2COOH, -$(CH_2)_3NHC(=NH)NH_2$, -$(CH_2)_3NH_2$, -$(CH_2)_3NHCOCH_3$, -$(CH_2)_3NHCHO$, -$(CH_2)_4NHC(=NH)NH_2$, -$(CH_2)_4NH_2$, -$(CH_2)_4NHCOCH_3$, -$(CH_2)_4NHCHO$, -$(CH_2)_3NHCONH_2$, -$(CH_2)_4NHCONH_2$, -$CH_2CH_2CH(OH)CH_2NH_2$, 2-pyridylmethyl-, 3-pyridylmethyl-, 4-pyridylmethyl-, phenyl, cyclohexyl,



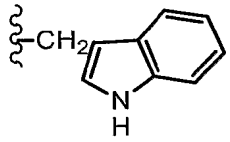
The amino acid unit of the linker unit can be enzymatically cleaved by an enzyme including, but not limited to, a tumor-associated protease to liberate the drug unit (-D) which is protonated *in vivo* upon release to provide a cytotoxic drug (D).

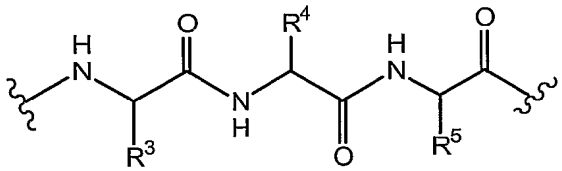
Illustrative W_w units are represented by formulas (**V**)-(**VII**):



(**V**)

wherein R^3 and R^4 are as follows:

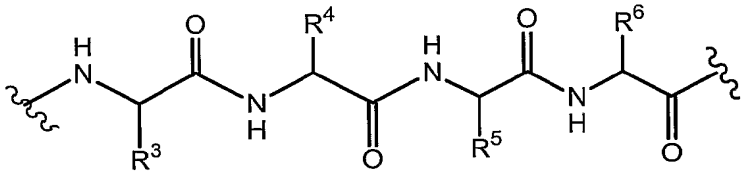
R^3	R^4
benzyl	$(CH_2)_4NH_2$;
methyl	$(CH_2)_4NH_2$;
isopropyl	$(CH_2)_4NH_2$;
isopropyl	$(CH_2)_3NHCONH_2$;
benzyl	$(CH_2)_3NHCONH_2$;
isobutyl	$(CH_2)_3NHCONH_2$;
sec-butyl	$(CH_2)_3NHCONH_2$;
	$(CH_2)_3NHCONH_2$;
benzyl	methyl; and
benzyl	$(CH_2)_3NHC(=NH)NH_2$;



(VI)

wherein R^3, R^4 and R^5 are as follows:

R^3	R^4	R^5
benzyl	benzyl	$(CH_2)_4NH_2$;
isopropyl	benzyl	$(CH_2)_4NH_2$; and
H	benzyl	$(CH_2)_4NH_2$;



(VII)

wherein R^3, R^4, R^5 and R^6 are as follows:

R^3	R^4	R^5	R^6
H	benzyl	isobutyl	H; and
methyl	isobutyl	methyl	isobutyl.

Preferred amino acid units include, but are not limited to, units of formula (V) where: R^3 is benzyl and R^4 is $-(CH_2)_4NH_2$; R^3 is isopropyl and R^4 is $-(CH_2)_4NH_2$; R^3 is isopropyl and R^4 is $-(CH_2)_3NHCONH_2$. Another preferred amino acid unit is a unit of formula (**VI**), where: R^3 is benzyl, R^4 is benzyl, and R^5 is $-(CH_2)_4NH_2$.

$-W_w-$ units useful in the present invention can be designed and optimized in their selectivity for enzymatic cleavage by a particular tumor-associated protease. The preferred $-W_w-$ units are those whose cleavage is catalyzed by the proteases, cathepsin B, C and D, and plasmin.

In one embodiment, $-W_w-$ is a dipeptide, tripeptide or tetrapeptide unit.

Where R^2, R^3, R^4, R^5 or R^6 is other than hydrogen, the carbon atom to which R^2, R^3, R^4, R^5 or R^6 is attached is chiral.

Each carbon atom to which R^2, R^3, R^4, R^5 or R^6 is attached is independently in the (S) or (R) configuration.

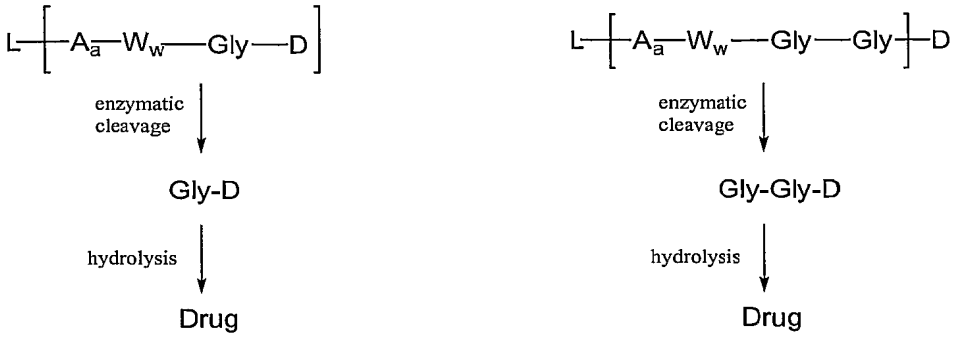
In a preferred embodiment, the amino acid unit is a phenylalanine-lysine dipeptide (phe-lys or FK linker). In antother preferred embodiment, the amino acid unit is a valine-citrulline dipeptide (val-cit or VC linker).

5.6.1.3 THE SPACER UNIT

The spacer unit (-Y-), when present, links an amino acid unit to the drug unit. Spacer units are of two general types: self-immolative and non self-immolative. A non self-immolative spacer unit is one in which part or all of the spacer unit remains bound to the drug unit after enzymatic cleavage of an amino acid unit from the anti-CD30 antibody-linker-drug conjugate or the drug-linker compound. Examples of a non self-immolative spacer unit include, but are not limited to a (glycine-glycine) spacer unit and a glycine spacer unit (both depicted in Scheme 1). When an anti-CD30 antibody-linker-drug conjugate of the invention containing a glycine-glycine spacer unit or a glycine spacer unit undergoes enzymatic cleavage via a tumor-cell associated-protease, a cancer-cell-associated protease or a lymphocyte-associated protease, a glycine-glycine-drug moiety or a glycine-drug moiety is cleaved from A-T-W_w-. To liberate the drug, an independent hydrolysis reaction should take place within the target cell to cleave the glycine-drug unit bond.

In a preferred embodiment, -Y_y- is a p-aminobenzyl ether which can be substituted with Q_m where Q is is -C_1-C_8 alkyl, -C_1-C_8 alkoxy, -halogen,- nitro or -cyano; and m is an integer ranging from 0-4.

Scheme 1



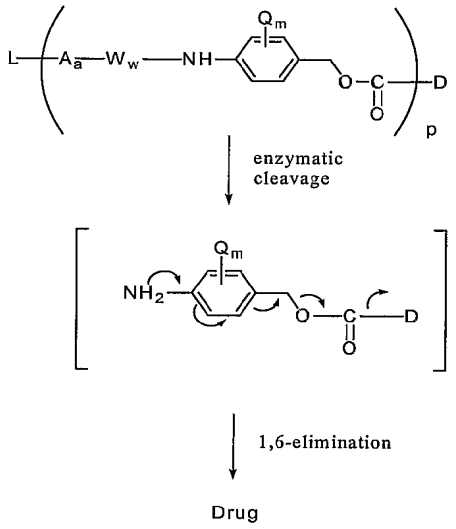
In one embodiment, a non self-immolative spacer unit (-Y-) is -Gly-Gly-.

In another embodiment, a non self-immolative the spacer unit (-Y-) is -Gly-.

In one embodiment, the drug-linker compound or an anti-CD30 antibody-linker-drug conjugate lacks a spacer unit (y=0).

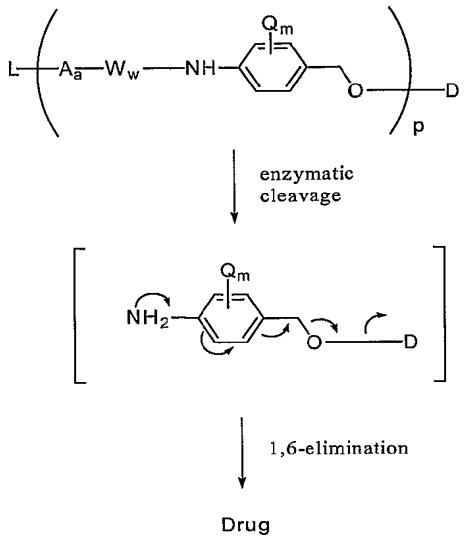
Alternatively, an anti-CD30 antibody-linker-drug conjugate of the invention containing a self-immolative spacer unit can release the drug (D) without the need for a separate hydrolysis step. In these embodiments, -Y- is a *p*-aminobenzyl alcohol (PAB) unit that is linked to -W_w- via the nitrogen atom of the PAB group, and connected directly to -D via a carbonate, carbamate or ether group (Scheme 2 and Scheme 3).

Scheme 2



where Q is -C_1-C_8 alkyl, -C_1-C_8 alkoxy, -halogen, -nitro or -cyano; m is an integer ranging from 0-4; and p is an integer ranging from 1-20.

Scheme 3

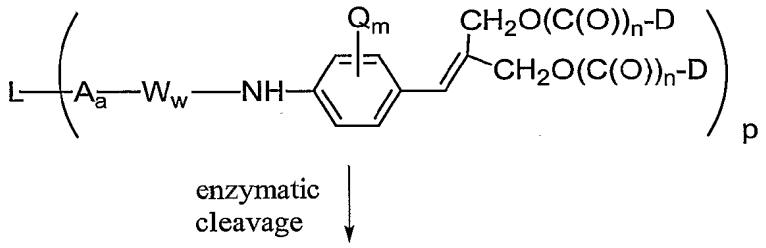


where Q is -C_1-C_8 alkyl, -C_1-C_8 alkoxy, -halogen,- nitro or -cyano; m is an integer ranging from 0-4; and p is an integer ranging from 1-20.

Other examples of self-immolative spacers include, but are not limited to, aromatic compounds that are electronically equivalent to the PAB group such a 2-aminoimidazol-5-methanol derivatives (see Hay *et al.*, *Bioorg. Med. Chem. Lett.*, 1999, 9, 2237 for examples) and ortho or para-aminobenzylacetals. Spacers can be used that undergo facile cyclization upon amide bond hydrolysis, such as substituted and unsubstituted 4-aminobutyric acid amides (Rodrigues *et al.*, *Chemistry Biology*, 1995, 2, 223), appropriately substituted bicyclo[2.2.1] and bicyclo[2.2.2] ring systems (Storm, *et al.*, *J. Amer. Chem. Soc.*, 1972, 94, 5815) and 2-aminophenylpropionic acid amides (Amsberry, *et al.*, *J. Org. Chem.*, 1990, 55, 5867). Elimination of amine-containing drugs that are substituted at the α-position of glycine (Kingsbury, *et al.*, *J. Med. Chem.*, 1984, 27, 1447) are also examples of self-immolative spacer strategies that can be applied to the anti-CD30 antibody-linker-drug conjugates of the invention.

In an alternate embodiment, the spacer unit is a branched bis(hydroxymethyl)styrene (BHMS) unit (Scheme 4), which can be used to incorporate additional drugs.

Scheme 4

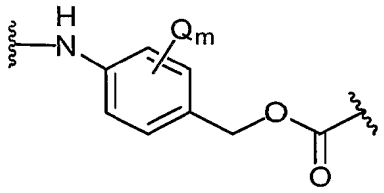


where Q is -C_1-C_8 alkyl, -C_1-C_8 alkoxy, -halogen, -nitro or -cyano; m is an integer ranging from 0-4; n is 0 or 1; and p is an integer raging from 1-20.

In one embodiment, the two -D moieties are the same.

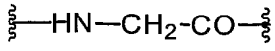
In another embodiment, the two -D moieties are different.

Preferred spacer units (-Y_y-) are represented by Formulas (**VIII**)-(**X**):

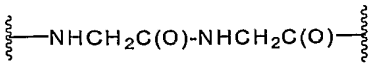


(**VIII**)

where Q is C_1-C_8 alkyl, C_1-C_8 alkoxy, halogen, nitro or cyano; and m is an integer ranging from 0-4;



(**IX**); and



(X).

5.6.2 DRUGS

The present invention encompasses the use of anti-CD30 ADCs for the treatment or prevention of an immunological disorder. As used herein, the term "drug" or "cytotoxic agent," where employed in the context of an anti-CD30 ADC of the invention, does not include radioisotopes. Otherwise, any drug that is known to the skilled artisan can be used in connection with the ADCs of the present invention.

The drugs used for conjugation to the anti-CD30 antibodies of the present invention can include conventional chemotherapeutics, such as doxorubicin, paclitaxel, melphalan, vinca alkaloids, methotrexate, mitomycin C, etoposide, and others. In addition, potent agents such CC-1065 analogues, calichiamicin, maytansine, analogues of dolastatin 10, rhizoxin, and palytoxin can be linked to the anti-CD30 antibodies using the conditionally stable linkers to form potent immunoconjugates. Examples of other suitable drugs for conjugation to the anti-CD30 antibodies of the present invention are provided in Section 5.12.1 below.

In certain embodiments, the ADCs of the invention comprise drugs that are at least 40-fold more potent than doxorubicin on CD30-expressing cells. Such drugs include, but are not limited to: DNA minor groove binders, including enediynes and lexitropsins, duocarmycins, taxanes (including paclitaxel and docetaxel), puromycins, vinca alkaloids, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, echinomycin, combretastatin, netropsin, epithilone A and B, estramustine, cryptophysins, cemadotin, maytansinoids, dolastatins, *e.g.*, auristatin E, dolastatin 10, MMAE, discodermolide, eleutherobin, and mitoxantrone.

In certain specific embodiments, an anti-CD30 ADC of the invention comprises an enediyne moiety. In a specific embodiment, the enediyne moiety is

calicheamicin. Enediyne compounds cleave double stranded DNA by generating a diradical via Bergman cyclization.

A variety of cytotoxic and cytostatic agents that can be used with the compositions and methods of the present invention are described in U.S. provisional application no. 60/400,403, entitled "Drug Conjugates and their use for treating cancer, an autoimmune disease or an infectious disease", by Inventors: Peter D. Senter, Svetlana Doronina and Brian E. Toki, filed on July 31, 2002, which is incorporated by reference in its entirety herein.

In other specific embodiments, the cytotoxic or cytostatic agent is auristatin E or a derivative thereof.

In preferred embodiments, the auristatin E derivative is an ester formed between auristatin E and a keto acid. For example, auristatin E can be reacted with paraacetyl benzoic acid or benzoylvaleric acid to produce AEB and AEVB, respectively. Other preferred auristatin derivatives include MMAE and AEFP.

The synthesis and structure of auristatin E, also known in the art as dolastatin-10, and its derivatives are described in U.S. Patent Application Nos.: 09/845,786 and 10/001,191; in the International Patent Application No.: PCT/US02/13435, in U.S. Patent Nos: 6,323,315; 6,239,104; 6,034,065; 5,780,588; 5,665,860; 5,663,149; 5,635,483; 5,599,902; 5,554,725; 5,530,097; 5,521,284; 5,504,191; 5,410,024; 5,138,036; 5,076,973; 4,986,988; 4,978,744; 4,879,278; 4,816,444; and 4,486,414, all of which are incorporated by reference in their entireties herein.

In specific embodiments, the drug is a DNA minor groove binding agent. Examples of such compounds and their syntheses are disclosed in U.S. Patent No.: 6,130,237, which is incorporated by reference in its entirety herein. In certain embodiments, the drug is a CBI compound.

In certain embodiments of the invention, an ADC of the invention comprises an anti-tubulin agent. Anti-tubulin agents are a well established class of cancer therapy compounds. Examples of anti-tubulin agents include, but are not limited to, taxanes (*e.g.*, Taxol® (paclitaxel), docetaxel), T67 (Tularik), vincas, and auristatins (*e.g.*, auristatin E, AEB, AEVB, MMAE, AEFP). Antitubulin agents included in this class are also: vinca alkaloids, including vincristine and vinblastine, vindesine and vinorelbine;

taxanes such as paclitaxel and docetaxel and baccatin derivatives, epithilone A and B, nocodazole, colchicine and colcimid, estramustine, cryptophysins, cemadotin, maytansinoids, combretastatins, dolastatins, discodermolide and eleutherobin.

In a specific embodiment, the drug is a maytansinoid, a group of anti-tubulin agents. In a more specific embodiment, the drug is maytansine. Further, in a specific embodiment, the cytotoxic or cytostatic agent is DM-1 (ImmunoGen, Inc.; see also Chari et al, 1992, Cancer Res 52:127-131). Maytansine, a natural product, inhibits tubulin polymerization resulting in a mitotic block and cell death. Thus, the mechanism of action of maytansine appears to be similar to that of vincristine and vinblastine. Maytansine, however, is about 200 to 1,000-fold more cytotoxic *in vitro* than these vinca alkaloids.

In another specific embodiment, the drug is an AEFP.

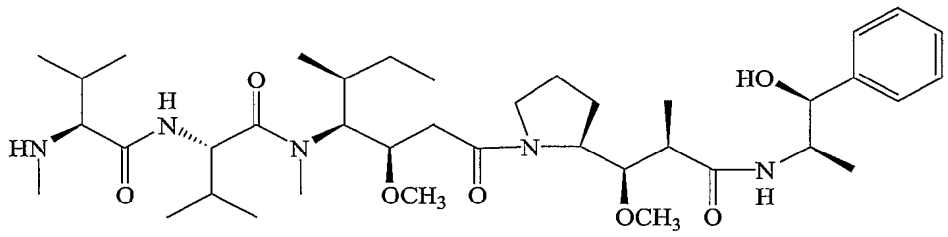
In certain specific embodiments of the invention, the drug is not a polypeptide of greater than 50, 100 or 200 amino acids, for example a toxin. In a specific embodiment of the invention, the drug is not ricin.

In other specific embodiments of the invention, an ADC of the invention does not comprise one or more of the cytotoxic or cytostatic agents the following non-mutually exclusive classes of agents: alkylating agents, anthracyclines, antibiotics, antifolates, antimetabolites, antitubulin agents, auristatins, chemotherapy sensitizers, DNA minor groove binders, DNA replication inhibitors, duocarmycins, etoposides, fluorinated pyrimidines, lexitropsins, nitrosoureas, platinols, purine antimetabolites, puromycins, radiation sensitizers, steroids, taxanes, topoisomerase inhibitors, vinca alkaloids, purine antagonists, and dihydrofolate reductase inhibitors. In more specific embodiments, the high potency drug is not one or more of an androgen, anthramycin (AMC), asparaginase, 5-azacytidine, azathioprine, bleomycin, busulfan, buthionine sulfoximine, camptothecin, carboplatin, carmustine (BSNU), CC-1065, chlorambucil, cisplatin, colchicine, cyclophosphamide, cytarabine, cytidine arabinoside, cytochalasin B, dacarbazine, dactinomycin (formerly actinomycin), daunorubicin, decarbazine, docetaxel, doxorubicin, an estrogen, 5-fluordeoxyuridine, 5-fluorouracil, gramicidin D, hydroxyurea, idarubicin, ifosfamide, irinotecan, lomustine (CCNU), mechlorethamine, melphalan, 6-mercaptopurine, methotrexate, mithramycin, mitomycin C, mitoxantrone, nitroimidazole,

paclitaxel, plicamycin, procarbizine, streptozotocin, tenoposide, 6-thioguanine, thioTEPA, topotecan, vinblastine, vincristine, vinorelbine, VP-16, VM-26, azothioprine, mycophenolate mofetil, methotrexate, acyclovir, gangcyclovir, zidovudine, vidarabine, ribavarin, azidothymidine, cytidine arabinoside, amantadine, dideoxyuridine, iododeoxyuridine, poscarnet, and trifluridine.

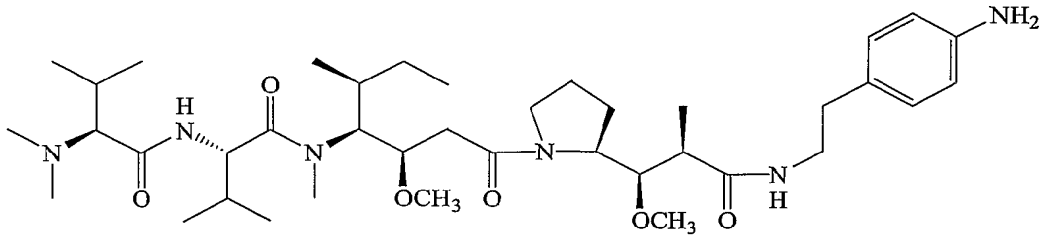
5.6.2.1 DOLASTATIN DRUGS

In certain embodiments, the cytotoxic or cytostatic agent is a dolastatin. In more specific embodiments, the dolastatin is of the auristatin class. In a specific embodiment of the invention, the cytotoxic or cytostatic agent is MMAE (MMAE; Formula **XI**). In another specific embodiment of the invention, the cytotoxic or cytostatic agent is AEFP (Formula **XVI**).

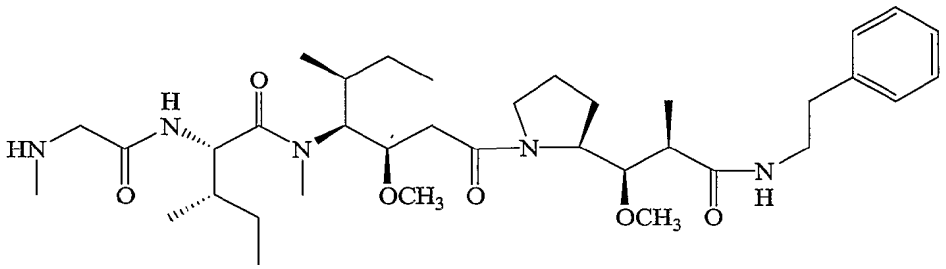


(XI)

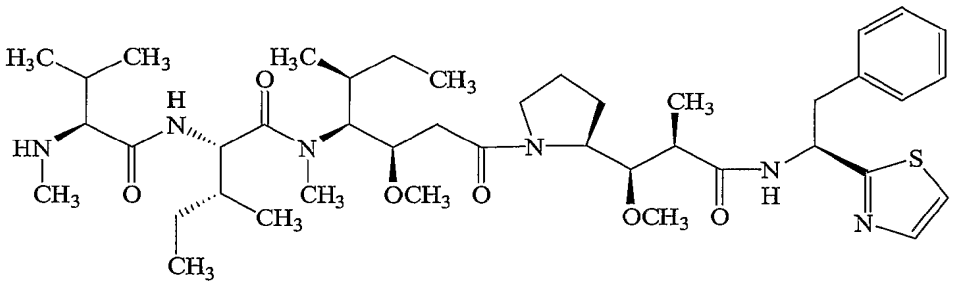
In certain embodiments of the invention, the cytotoxic or cytostatic agent is a dolastatin of formulas **XII-XVIII.**



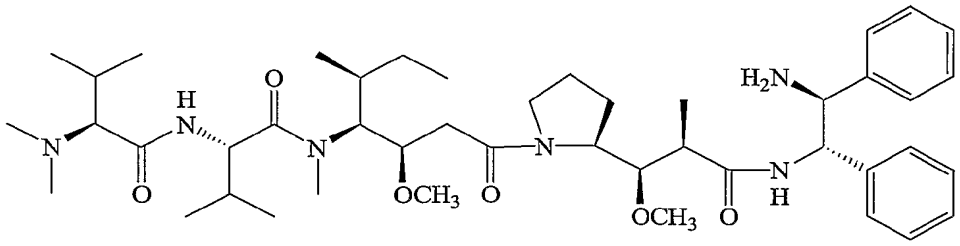
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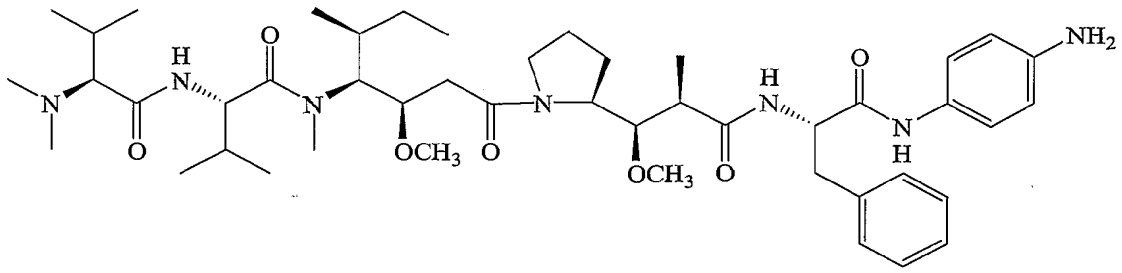
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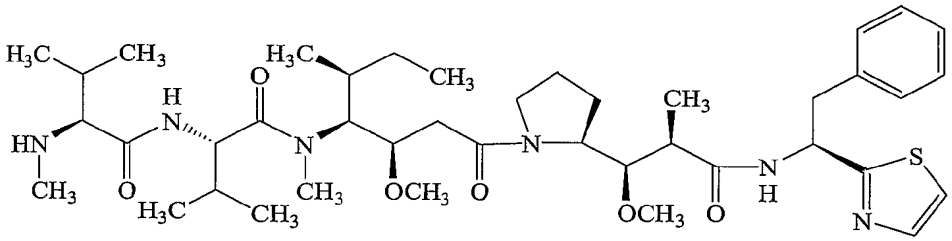
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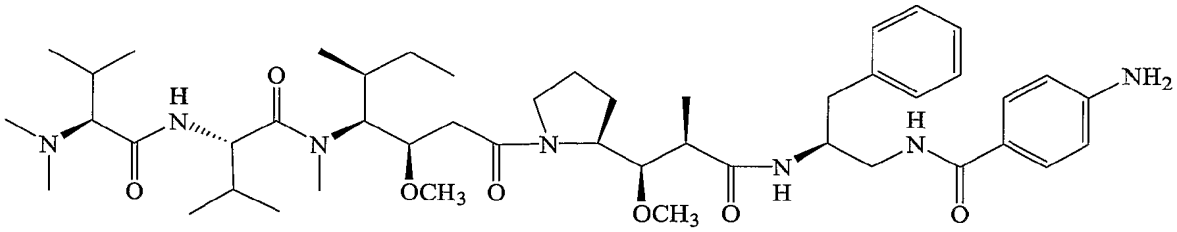
(XV)



(XVI)



(XVII)



(XVIII)

5.6.3 FORMATION OF ANTI-CD30 ANTIBODY-DRUG CONJUGATES

The generation of anti-CD30 antibody drug conjugates (ADCs) can be accomplished by any technique known to the skilled artisan. Briefly, the anti-CD30 ADCs comprise an anti-CD30 antibody, a drug, and a linker that joins the drug and the antibody. A number of different reactions are available for covalent attachment of drugs to antibodies. This is often accomplished by reaction of the amino acid residues of the antibody molecule, including the amine groups of lysine, the free carboxylic acid groups of glutamic and aspartic acid, the sulfhydryl groups of cysteine and the various moieties of the aromatic amino acids. One of the most commonly used non-specific methods of covalent attachment is the carbodiimide reaction to link a carboxy (or amino) group of a compound to amino (or carboxy) groups of the antibody. Additionally, bifunctional agents such as dialdehydes or imidoesters have been used to link the amino group of a compound to amino groups of the antibody molecule. Also available for attachment of drugs to antibodies is the Schiff base reaction. This method involves the periodate oxidation of a drug that contains glycol or hydroxy groups, thus forming an aldehyde which is then reacted with the antibody molecule. Attachment occurs via formation of a Schiff base with amino groups of the antibody molecule. Isothiocyanates can also be used as coupling agents for covalently attaching drugs to antibodies. Other techniques known to the skilled artisan and within the scope of the present invention. Non-limiting examples of such techniques are described in, *e.g.*, U.S. Patent Nos. 5,665,358, 5,643,573, and 5,556,623, which are incorporated by reference in their entireties herein.

In certain embodiments, an intermediate, which is the precursor of the linker, is reacted with the drug under appropriate conditions. In certain embodiments, reactive groups are used on the drug and/or the intermediate. The product of the reaction between the drug and the intermediate, or the derivatized drug, is subsequently reacted with the anti-CD30 antibody under appropriate conditions. Care should be taken to maintain the stability of the antibody under the conditions chosen for the reaction between the derivatized drug and the antibody.

5.7 ASSAYS FOR CYTOTOXIC AND CYTOSTATIC ACTIVITIES

By definition, a protein of the invention must exert a cytostatic or cytotoxic effect on an activated lymphocyte. Activated lymphocytes that can be assayed for a cytostatic or cytotoxic effect of a CD30 binding protein may be cultured cell lines (*e.g.*, Jurkat and CESS, both of which are available from the ATCC; or Karpas 299 and L540, both of which are available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), or from lymphocytes prepared from a fresh blood sample. Lymphocytes can be activated by the appropriate cocktails of antibodies and cytokines, as will be recognized by one of skill in the art. For example, T lymphocytes can be activated using a combination anti-CD3 and anti-CD28 antibodies and IL-2, as described in Section 11 below.

Many methods of determining whether a protein exerts a cytostatic or cytotoxic effect on a cell are known to those of skill in the art, and can be used to elucidate whether a particular protein is a protein of the invention. Illustrative examples of such methods are described below.

Wherein a protein that binds to CD30 does not exert a cytostatic or cytotoxic effect on activated lymphocytes, the protein can be multimerized according to the methods described in Section 5.1, *supra*, and the multimer assayed for its ability to exert a cytostatic or cytotoxic effect on activated lymphocytes.

In a preferred embodiment, the proteins of the invention are cross-linked prior to assessing their cytotoxic or cytostatic effect on activated lymphocytes. In an exemplary embodiment, in which the protein of the invention is an anti-CD30 antibody, the antibody can be cross-linked in solution, and one or more dilutions of the anti-CD30 antibody can be titrated into 96-well flat bottom tissue culture plates in the absence or presence of secondary antibodies. Activated lymphocytes are then added to the plates at approximately 5,000 cells/well. The cytostatic or cytotoxic effect can then be assessed as described herein, for example as an inhibition of radiolabeled thymidine incorporation into the activated lymphocytes.

Once a protein is identified that both (i) binds to CD30 and (ii) exerts a cytostatic or cytotoxic effect on activated lymphocytes, its therapeutic value is validated

in an animal model. Exemplary animal models of immunological disorders are described in Section 5.7.1, *infra.*

In a preferred embodiment for determining whether an anti-CD30 antibody exerts a cytostatic effect on activated lymphocytes, a thymidine incorporation assay may be used. For example, activated lymphocytes at a density of 5,000 cells/well of a 96-well plated can be cultured for a 72-hour period and exposed to 0.5 μCi of ^{3}H-thymidine during the final 8 hours of the 72-hour period, and the incorporation of ^{3}H-thymidine into cells of the culture is measured in the presence and absence of the antibody.

There are many cytotoxicity assays known to those of skill in the art. Some of these assays measure necrosis, while others measure apoptosis (programmed cell death). Necrosis is accompanied by increased permeability of the plasma membrane; the cells swell and the plasma membrane ruptures within minutes. On the other hand, apoptosis is characterized by membrane blebbing, condensation of cytoplasm and the activation of endogenous endonucleases. Only one of these effects on activated lymphocytes is sufficient to show that a CD30-binding protein is useful in the treatment or prevention of activated lymphocytes as an alternative to the assays measuring cytostatic or cytotoxic effects described above.

In one embodiment, necrosis measured by the ability or inability of a cell to take up a dye such as neutral red, trypan blue, or ALAMAR™ blue (Page *et al.*, 1993, Intl. J. of Oncology 3:473-476). In such an assay, the cells are incubated in media containing the dye, the cells are washed, and the remaining dye, reflecting cellular uptake of the dye, is measured spectrophotometrically.

In another embodiment, the dye is sulforhodamine B (SRB), whose binding to proteins can be used as a measure of cytotoxicity (Skehan *et al.*, 1990, J. Nat'l Cancer Inst. 82:1107-12).

In yet another embodiment, a tetrazolium salt, such as MTT, is used in a quantitative colorimetric assay for mammalian cell survival and proliferation by detecting living, but not dead, cells (*see*, *e.g.*, Mosmann, 1983, J. Immunol. Methods 65:55-63).

In yet another embodiment, apoptotic cells are measured in both the attached and "floating" compartments of the cultures. Both compartments are collected by removing the supernatant, trypsinizing the attached cells, and combining both

preparations following a centrifugation wash step (10 minutes, 2000 rpm). The protocol for treating tumor cell cultures with sulindac and related compounds to obtain a significant amount of apoptosis has been described in the literature (*see, e.g.*, Piazza *et al.*, 1995, Cancer Research 55:3110-16). Features of this method include collecting both floating and attached cells, identification of the optimal treatment times and dose range for observing apoptosis as detected by DNA fragmentation, and identification of optimal cell culture conditions.

In yet another embodiment, apoptosis is quantitated by measuring DNA fragmentation. Commercial photometric methods for the quantitative *in vitro* determination of DNA fragmentation are available. Examples of such assays, including TUNEL (which detects incorporation of labeled nucleotides in fragmented DNA) and ELISA-based assays, are described in Biochemica, 1999, no. 2, pp. 34-37 (Roche Molecular Biochemicals).

In yet another embodiment, apoptosis can be observed morphologically. Following treatment with a test protein or nucleic acid, cultures can be assayed for apoptosis and necrosis by fluorescence microscopy following labeling with acridine orange and ethidium bromide. The method for measuring apoptotic cell number has previously been described by Duke & Cohen, 1992, Current Protocols In Immunology, Coligan *et al.*, eds., 3.17.1-3.17.16. In another mode of the embodiment, cells can be labeled with the DNA dye propidium iodide, and the cells observed for morphological changes such as chromatin condensation and margination along the inner nuclear membrane, cytoplasmic condensation, increased membrane blebbing and cellular shrinkage.

5.7.1 ANIMAL MODELS OF IMMUNOLOGICAL DISORDERS

The molecules of the invention can be tested or validated in animal models of immunological disorders before they are subjected to clinical testing. A number of established animal models of immunological disorders are known to the skilled artisan, any of which can be used to assay the efficacy of the molecules of the invention. Non-limiting examples of such models are described below.

Some examples for animal models of systemic and organ-specific autoimmune diseases including diabetes, lupus, systemic sclerosis, Sjögren's Syndrome, experimental autoimmune encephalomyelitis (multiple sclerosis), thyroiditis, myasthenia gravis, arthritis, uveitis, inflammatory bowel disease have been described by Bigazzi, P., "Animal Models of Autoimmunity: Spontaneous and Induced", in The Autoimmune Diseases, Rose and Mackay (eds.), pp.211-244 (Academic Press, 1998) and in "Animal Models for Autoimmune and Inflammatory Disease", in Current Protocols in Immunology, Coligan et al. (eds.), Chapter 15 (Wiley, 1997).

Allergic conditions, *e.g.*, asthma and dermatitis, can also be modeled in rodents. Airway hypersensitivity can be induced in mice by ovalbumin (Tomkinson *et al.*, 2001, J. Immunol. 166:5792-5800) or Schistosoma mansoni egg antigen (Tesciuba *et al.*, 2001, J. Immunol. 167:1996-2003). The Nc/Nga strain of mice show marked increase in serum IgE and spontaneously develop atopic dermatitis-like leisons (Vestergaard *et al.*, 2000, Mol. Med. Today 6:209-210; Watanabe *et al.*, 1997, Int. Immunol. 9:461-466; Saskawa *et al.*, 2001, Int. Arch. Allergy Immunol. 126:239-247).

Injection of immuno-competent donor lymphocytes into a lethally irradiated histo-incompatible host is a classical approach to induce acute GVHD in mice. Alternatively, the parent→B6D2F1 murine model provides a system to induce both acute and chronic GVHD. In this model the B6D2F1 mice are F1 progeny from a cross between the parental strains of C57BL/6 and DBA/2 mice. Transfer of DBA/2 lymphoid cells into non-irradiated B6D2F1 mice causes chronic GVHD, whereas transfer of C57BL/6, C57BL/10 or B10.D2 lymphoid cells causes acute GVHD (Slayback *et al.*, 2000, Bone Marrow Transpl. 26: 931-938; Kataoka *et al.*, 2001, Immunology 103:310-318).

Additionally, both human hematopoietic stem cells and mature peripheral blood lymphoid cells can be engrafted into SCID mice, and these human lympho-hematopoietic cells remain functional in the SCID mice (McCune *et al.*, 1988, Science 241:1632-1639; Kamel-Reid and Dick, 1988, Science 242:1706-1709; Mosier *et al.*, 1988, Nature 335:256-259). This has provided a small animal model system for the direct testing of potential therapeutic agents on human lymphoid cells. For example, a human-mouse chimera model has been applied to examine the therapeutic potentials of

anti-IL-4, anti-IL-13, anti-IL-5, and the double-mutein IL-4 (Tournoy *et al.*, 2001, J. Immunol. 166:6982-6991).

5.8 ASSAYS FOR SIGNALING ACTIVITY

In certain preferred embodiments, a protein of the invention is capable of inducing one or more hallmarks of signaling through CD30 upon binding to a CD30-expressing lymphocyte. CD30-expressing lymphocytes that can be assayed for a signaling effect of a CD30 binding protein may be cultured cell lines (*e.g.*, Jurkat and CESS, both of which are available from the ATCC; or Karpas 299 and L540, both of which are available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH), or lymphocytes prepared from a fresh blood sample.

In a preferred embodiment, the proteins of the invention are cross-linked prior to assessing their activity on activated lymphocytes. In an exemplary embodiment, where the protein of the invention is an anti-CD30 antibody, the anti-CD30 antibody can be cross-linked in solution. Briefly, one or more dilutions of the anti-CD30 antibody can be titrated into 96-well flat bottom tissue culture plates in the absence or presence of secondary antibodies. Lymphocytes are then added to the plates at approximately 5,000 cells/well. The signaling activity of the antibody can then be assessed as described herein.

Many methods of determining whether a protein induces one or more hallmarks of signaling through CD30 are known to those of skill in the art. Illustrative examples of such methods are described below.

5.8.1 CALCIUM RELEASE

In one embodiment, a protein of the invention can induce the release of intracellular free Ca^{2+} in Jurkat cells when it is cross-linked, for example with a secondary antibody. The release of intracellular free Ca^{2+} can be measured as described by Ellis *et al.* (1993, J. Immunol., 151, 2380-2389) or by Mond and Brunswick (1998, Current Protocols in Immunology, Unit 3.9, Wiley).

5.8.2 TRAF LOCALIZATION

Four TNF receptor-associated factors (TRAFs) including TRAF1, TRAF2, TRAF3, and TRAF5 have been demonstrated to interact with the cytoplasmic tail of CD30 (Gedrich *et al.*, 1996, J. Biol. Chem., 271, 12852-12858; Lee *et al.*, 1996, Proc. Natl. Acad. Sci. USA., 93, 9699-9703; Ansieau *et al.*, 1996, Proc. Natl. Acad. Sci. USA., 93, 14053-14058; Aizawa *et al.*, 1997, J. Biol. Chem., 272, 2042-2045; Tsitsikov *et al.*, 1997, Proc. Natl. Acad. Sci. USA., 94, 1390-1395; Lee *et al.*, 1997, J. Exp. Med., 185, 1275-1285; Duckett and Thompson, 1997, Genes Dev., 11, 2810-2821). Using co-transfection studies, yeast two-hybrid screening, and GST fusion proteins, the TRAF interacting sites have been mapped to the carboxyl terminal of the cytoplasmic tail of CD30, and the association between CD30 and the TRAFs in the cytosolic phase has been hypothesized to be a key event in the CD30-mediated signal cascade. The interaction between CD30 and TRAF does not appear to require CD30 ligation (Ansieau *et al.*, 1996, Proc. Natl. Acad. Sci. USA., 93, 14053-14058; Aizawa *et al.*, 1997, J. Biol. Chem., 272, 2042-2045). However, cross-linking of CD30 leads to a disappearance of TRAF1 and TRAF2 from the detergent-soluble fractions of cell lysates (Duckett and Thompson, 1997, Genes Dev., 11, 2810-2821; Arch *et al.*, 2000, Biochem. Biophys. Res. Commun., 272, 936-945). The disappearance of TRAF2 is accompanied by a corresponding increase in the quantity of TRAF2 detectable in the detergent-insoluble fraction containing the nuclei (Arch *et al.*, 2000, Biochem. Biophys. Res. Commun., 272, 936-945). Further subcellular localization studies have confirmed that cross-linking of CD30 induces a translocation of TRAF2 from the cytosol to the perinuclear region of cells (Arch *et al.*, 2000, Biochem. Biophys. Res. Commun., 272, 936-945). Such CD30-mediated translocation of TRAF2 is hypothesized to modulate cell survival by regulating the sensitivity of cells to undergo apoptosis induced by other TRAF-binding members of the TNF receptor superfamily (Duckett and Thompson, 1997, Genes Dev., 11, 2810-2821; Arch *et al.*, 2000, Biochem. Biophys. Res. Commun., 272, 936-945).

To determine whether an antibody of the invention induces nuclear translocation of TRAF2, the antibody of the invention is contacted with CD30+ cells and a cross-linking agent, such as a secondary antibody. Confocal microscopy can then be used to compare localization of TRAF2 in cells incubated with the antibody of the

invention (plus cross-linking reagent) versus cells not incubated with the antibody of the invention.

In an alternative embodiment, whether an antibody of the invention induces TRAF2 nuclear localization can be assayed by measuring the amount of TRAF2 in various cell fractions, for example on a Western Blot. For example, 2 μg/ml of an antibody of the invention can be incubated with $CD30^+$ cells at 0.5 x 10^6/ml. The antibody is cross-linked by 20 μg/ml of a secondary antibody (*e.g.*, where the antibody of the invention is a mouse monoclonal antibody, a goat anti-mouse IgG Fc specific antibody (Jackson ImmunoReseach, West Grove, PA) can be used as a secondary antibody) at 37°C and 5% CO_2. At designated time-points (*e.g.*, 2 to 24 hours), 5 x 10^6 cells are removed and spun down. After two washes with ice-cold PBS, cells are lysed at 100 x 10^6/ml in a lysis buffer (0.15 M NaCl, 0.05 M Tris-HCl, pH 8.0, 0.005 M EDTA, and 0.5% NP-40 or Triton X-100) supplemented with a protease inhibitor cocktail (Roche Diagnositc GmBH, Mannheim, Germany). Lysis is done at 4°C for 2 hours with constant mixing. After lysis, the detergent-soluble and detergent-insoluble fractions are separated by centrifugation at 14,000 x g for 20 minutes. The detergent-soluble fraction is then transferred to a separate tube and an equal volume of 2X SDS-PAGE reducing sample buffer is added to it. An equal volume of 1X SDS-PAGE reducing sample buffer is also added to the detergent-insoluble fraction, *i.e.*, the pellet after centrifugation. Both fractions are heated to 100°C for 2 minutes. About 10 μl of the fractions from each time point is then resolved by 12% Tris-glycine SDS-PAGE (Invitrogen, Carlsbad, CA). Resolved proteins are Western-transferred onto PVDF membranes (Invitrogen), which is blocked with Tris buffer saline (0.05 M Tris-HCl, pH 8.0, 0.138 M NaCl, 0.0027 M KCl) supplemented with 0.05% Tween 20 and 5% BSA. The blots are immunoblotted with an anti-TRAF2 antibody (Santa Cruz, San Diego, CA). The presence of TRAF2 protein in the different fractions is detected by horseradish peroxidase (HRP)-conjugated $F(ab')_2$ goat anti-rabbit IgG Fc (Jackson ImmunoResearch) and the peroxidase substrate kit DAB (Vector Laboratories, Burlingame, CA). Alternatively, the SuperSignal West Pico Chemiluminescent Substrate kit (Pierce, Rockford, IL) can also be used for detection.

5.8.3 NF-κB ACTIVATION

Another well-defined signal transduction event that can be induced by certain antibodies of the invention is the activation of NF-κB. Anti-CD30 mAbs including M44, M67, and Ber-H2 can activate NF-κB as detected by standard mobility shift DNA-binding assay (McDonald *et al.*, 1995, Eur. J. Immunol., 25, 2870-2876; Ansieau *et al.*, 1996, Proc. Natl. Acad. Sci. USA., 93, 14053-14058; Horie *et al.*, 1998, Int. Immunol., 10, 203-210). Such effect can be observed in Hodgkin cells, T cells, and transfectant expressing CD30 (McDonald *et al.*, 1995, Eur. J. Immunol., 25, 2870-2876; Biswas *et al.*, 1995, Immunity, 2, 587-596; Ansieau *et al.*, 1996, Proc. Natl. Acad. Sci. USA., 93, 14053-14058; Horie *et al.*, 1998, Int. Immunol., 10, 203-210). Initial mapping studies revealed that the interaction between TRAF1, TRAF2, and TRAF5 with the cytoplasmic tail of CD30 was required for the CD30-mediated activation of NF-κB (Lee *et al.*, 1996, Proc. Natl. Acad. Sci. USA., 93, 9699-9703; Ansieau *et al.*, 1996, Proc. Natl. Acad. Sci. USA., 93, 14053-14058; Aizawa *et al.*, 1997, J. Biol. Chem., 272, 2042-2045; Duckett *et al.*, 1997, Mol. Cell. Biol., 17, 1535-1542). More recently, evidence has become available that ligation of CD30 by agonistic mAbs can also activate NF-κB via a TRAF2/5-independent pathway (Horie *et al.*, 1998, Int. Immunol., 10, 203-210). Some of the biological consequences of the CD30-mediated activation of NF-κB include activation of gene transcription (Biswas *et al.*, 1995, Immunity, 2, 587-596; Maggi *et al.*, 1995, Immunity, 3, 251-255) and regulation of cell survival (Mir *et al.*, 2000, Blood, 96, 4307-4312; Horie *et al.*, 2002, Oncogene, 21, 2439-2503). Any of these characteristics of NF-κB activation can be assayed to determine whether an antibody of the invention induces one or more hallmarks of CD30 signaling.

Whether NF-κB activation is induced in $CD30^+$ cells by an antibody of the invention can be measured by, for example, incubating $CD30^+$ cells at 3×10^6/ml with the antibody at 2 μg/ml, the antibody then cross-linked (*e.g.*, where the antibody is a mouse monoclonal antibody, the antibody can be cross-linked by 20 μg/ml of a goat anti-mouse IgG Fc specific antibody (Jackson ImmunoReseach, West Grove, PA)) and the culture incubated at 37°C and 5% CO_2 for 1 hour with constant shaking. The cell density is adjusted to 1.2×10^6/ml, and incubation with shaking is carried on for an additional hour. Thereafter, cell density is further reduced to 0.6×10^6/ml, and cells are incubated for an

additional 46 hours at 37°C and 5% CO_2 without any further shaking. At the end of incubation, nuclear extracts can be prepared from stimulated cells and analyzed for NF-κB activation.

NF-κB activation is assayed by collecting the cells by centrifugation at 1850 x g for 20 minutes and then washing them once in 5 packed cell volumes of PBS. The cell pellet is resuspended in 5 packed cell volumes of a hypotonic buffer (0.01 M Hepes, pH 7.9, 0.0015 M $MgCl_2$, 0.01 M KCl, 0.0002 M phenylmethyl sulphonyl fluoride, 0.0005 M dithiothreitol). Cells are collected by centrifugation at 1850 x g for 5 minutes. The pellet is then resuspended in 3 packed cell volumes of the hypotonic buffer and allowed to swell on ice for 10 minutes. After that, swollen cells are homogenized with slow up-and-down strokes in a Dounce homogenizer, using a tight B pestle. Cell lysis is monitored by trypan blue exclusion, and enough strokes should be applied to achieve more than 80% cell lysis. The nuclei are pelleted by centrifugation at 3300 x g for 15 minutes. The supernatant (cytoplasmic extract) is removed. The nuclear pellet is then resuspended in ½ packed nuclei volume of a low-salt buffer (0.02 M Hepes, pH 7.9, 25% volume/volume glycerol, 0.0015 M $MgCl_2$, 0.02 M KCl, 0.0002 M EDTA, 0.0002 M phenylmethyl sulphonyl fluoride, 0.0005 M dithiothreitol). An equal volume of a high-salt buffer (0.02 M Hepes, pH 7.9, 25% volume/volume glycerol, 0.0015 M $MgCl_2$, 1.2 M KCl, 0.0002 M EDTA, 0.0002 M phenylmethyl sulphonyl fluoride, 0.0005 M dithiothreitol) is then slowly added to the nuclei suspension with gentle stirring to give a final KCl concentration of roughly 0.3 M. The extraction is allowed to continue for 30 minutes with gentle stirring. After extraction, the nuclei are removed by centrifugation at 25,000 x g for 30 minutes. The nuclear extraction is then dialyzed against 50 volumes of a dialysis buffer (0.02 M Hepes, pH 7.9, 20% volume/volume glycerol, 0.1 M KCl, 0.0002 M EDTA, 0.0002 M phenylmethyl sulphonyl fluoride, 0.0005 M dithiothreitol) until the conductivity of the nuclear extract is the same as the dialysis buffer. The nuclear extract is centrifuged once more at 25,000 x g for 20 minutes to remove residual debris, and the protein concentration of the supernatant is determined by the micro-BCA assay (Pierce).

The presence of NF-κB in nuclear extract of anti-CD30 stimulated cells can be detected by standard mobility shift DNA-binding assay using the Gel Shift Assay System (Promega, Madison, WI). A double stranded oligonucleotide probe containing a

consensus NF-κB binding motif with the sequence 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (SEQ ID NO:33) (Lenardo and Baltimore, 1989, Cell, 58, 227-229) is used as the specific probe to detect NF-κB in nuclear extracts. This probe is phosphorylated by T4 polynucleotide kinase and [α-^{32}P]ATP. The phosphorylated probe is purified by Sepharose G25 spin columns equilibrated with TE buffer (0.01 M Tris-HCl, pH 8.0, 0.001 M EDTA). Purified probed is then precipitated with ammonium acetate and ethanol and then resuspended in 100 µl of TE buffer. Reaction mixtures containing nuclear extracts from anti-CD30-treated cells and control-treated cells are separately combined with the Gel Shift Binding buffer, water and unlabeled competitor probes according to the manufacturers instruction. An unlabeled oligonucleotide containing the NF-κB consensus and an unlabeled irrelevant oligonucleotide are included in the reaction mixture as the sequence-specific and sequence-nonspecific competitors. After incubation for 10 minutes at room temperature, 1 µl of the ^{32}P-labeled NF-κB consensus oligonucleotide is added to each reaction. The reactions are allowed to continue for an additional 20 minutes at room temperature. At the end of the incubation, 1 µl of a 10X loading buffer (0.25M Tris-HCl, pH 7.5, 40% volume/volume glycerol, 0.2% bromophenol blue) is added to the reactions. The reactions are then loaded into individual wells of a 6% DNA retardation gel (Invitrogen) and resolved at 100 volt for 90 minutes in 0.5X TBE (0.045M Tris-HCl, 0.045 M boric acid, 0.001M EDTA). After electrophoresis, the gel is covered with plastic wrap and exposed to X-ray film at -70°C to detect the specific interaction between NF-κB and the oligonucleotide containing the NF-κB binding sequence.

5.9 IMMUNE DISORDERS

The methods of the present invention are useful for treating or preventing an immunological disorder, wherein the immunological disorder is characterized by inappropriate activation of lymphocytes. As used herein, the phrase "immunological disorder" does not encompass immunological cancers such as Hodgkin's Disease and anaplastic large cell lymphoma. Treatment or prevention of an immunological disorder, according to the methods of the present invention, is achieved by administering to a patient in need of such treatment or prevention a protein, preferably an antibody, that

binds to activated lymphocytes that are associated with the disease state and exerts a cytotoxic or cytostatic effect on the lymphocytes.

Examples of diseases that can be treated or prevented by the methods of the present invention include, but are not limited to, rheumatoid arthritis, multiple sclerosis, endocrine ophthalmopathy, uveoretinitis, systemic lupus erythematosus, myasthenia gravis, Grave's disease, glomerulonephritis, autoimmune hepatological disorder, autoimmune inflammatory bowel disease, anaphylaxis, allergic reaction, Sjogren's syndrome, juvenile onset (Type I) diabetes mellitus, primary biliary cirrhosis, Wegener's granulomatosis, fibromyalgia, inflammatory bowel disease, polymyositis, dermatomyositis, multiple endocrine failure, Schmidt's syndrome, autoimmune uveitis, Addison's disease, adrenalitis, thyroiditis, Hashimoto's thyroiditis, autoimmune thyroid disease, pernicious anemia, gastric atrophy, chronic hepatitis, lupoid hepatitis, atherosclerosis, presenile dementia, demyelinating diseases, subacute cutaneous lupus erythematosus, hypoparathyroidism, Dressler's syndrome, autoimmune thrombocytopenia, idiopathic thrombocytopenic purpura, hemolytic anemia, pemphigus vulgaris, pemphigus, dermatitis herpetiformis, alopecia arcata, pemphigoid, scleroderma, progressive systemic sclerosis, CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia), adult onset diabetes mellitus (Type II diabetes), male and female autoimmune infertility, ankylosing spondolytis, ulcerative colitis, Crohn's disease, mixed connective tissue disease, polyarteritis nedosa, systemic necrotizing vasculitis, juvenile onset rheumatoid arthritis, atopic dermatitis, atopic rhinitis, Goodpasture's syndrome, Chagas' disease, sarcoidosis, rheumatic fever, asthma, recurrent abortion, anti-phospholipid syndrome, farmer's lung, erythema multiforme, post cardiotomy syndrome, Cushing's syndrome, autoimmune chronic active hepatitis, bird-fancier's lung, allergic encephalomyelitis, toxic epidermal necrolysis, Alport's syndrome, alveolitis, allergic alveolitis, fibrosing alveolitis, interstitial lung disease, erythema nodosum, pyoderma gangrenosum, transfusion reaction, leprosy, malaria, leishmaniasis, trypanosomiasis, Takayasu's arteritis, polymyalgia rheumatica, temporal arteritis, schistosomiasis, giant cell arteritis, ascariasis, aspergillosis, Sampter's syndrome, eczema, lymphomatoid granulomatosis, Behcet's disease, Caplan's syndrome, Kawasaki's disease, dengue, encephalomyelitis, endocarditis, endomyocardial fibrosis, endophthalmitis,

erythema elevatum et diutinum, psoriasis, erythroblastosis fetalis, eosinophilic faciitis, Shulman's syndrome, Felty's syndrome, filariasis, cyclitis, chronic cyclitis, heterochronic cyclitis, Fuch's cyclitis, IgA nephropathy, Henoch-Schonlein purpura, graft versus host disease, transplantation rejection, human immunodeficiency virus infection, echovirus infection, cardiomyopathy, Alzheimer's disease, parvovirus infection, rubella virus infection, post vaccination syndromes, congenital rubella infection, Eaton-Lambert syndrome, relapsing polychondritis, cryoglobulinemia, Waldenstrom's macroglobulemia, Epstein-Barr virus infection, mumps, Evan's syndrome, and autoimmune gonadal failure.

Accordingly, the methods of the present invention encompass treatment of disorders of B lymphocytes (*e.g.*, systemic lupus erythematosus, Goodpasture's syndrome, rheumatoid arthritis, and type I diabetes), Th_1-lymphocytes (*e.g.*, rheumatoid arthritis, multiple sclerosis, psoriasis, Sjorgren's syndrome, Hashimoto's thyroiditis, Grave's disease, primary biliary cirrhosis, Wegener's granulomatosis, tuberculosis, or acute graft versus host disease), and Th_2-lymphocytes (*e.g.*, atopic dermatitis, systemic lupus erythematosus, atopic asthma, rhinoconjunctivitis, allergic rhinitis, Omenn's syndrome, systemic sclerosis, or chronic graft versus host disease).

An alternative way of classifying immunological disease states is by the underlying biological mechanism. The present invention is directed to treatment and prevention of immunological diseases arising by any of the following mechanisms, which are classified into four types:

Anaphylactic reactions. These reactions are mediated by IgE antibodies which bind to receptors on mast cells. When cross-linking occurs with antigens, the IgE antibodies stimulate the mast cells to release a number of pharmacologically active substances that can cause the symptoms characteristic of anaphylaxis. These reactions to antigenic challenge are immediate and potentially life-threatening. Examples of anaphylactic responses include, but are not limited to, allergic rhinitis, gastrointestinal allergy, atopic dermatitis, bronchial asthma and equine heaves and laminitis.

Cytotoxic (cytolytic) reactions. These cell surface reactions result from an interaction of antigen with IgM and/or IgG which activates the complement cascade, leading to the destruction of the cell. Examples of cytolytic reactions include, but are not limited to, leukocytopenia, hemolytic disease of newborn and Goodpasture's disease.

Autoimmune disorders that involve cytotoxic/cytolytic reactions are hemolytic anemia, thrombocytopenia and thyroiditis.

Immune complex reactions. Immune complex reactions occur when large complexes of antigen and IgG or IgM accumulate in the circulation or in tissue, fixing complement. Granulocytes are attracted to the site of complement fixation and release damaging lytic enzymes from their granules. An example of this type of reaction is serum sickness. Autoimmune disorders that involve immune complex reactions include systemic lupus erythrematosus, chronic glomerulonephritis and rheumatoid arthritis.

Cell-mediated immunity (CMI) reaction, or delayed-type hypersensitivity (DTH). In contrast to the first three types of immune responses, this hypersensitivity response is mediated by T lymphocytes rather than antibodies produced by B lymphocytes. Activated T lymphocytes release cytokines which can result in the accumulation and activation of macrophages, K cells and NK cells, which cause local tissue damage. This reaction can occur 1-2 days after antigenic challenge.

5.10 GENE THERAPY

In a specific embodiment, nucleic acids encoding proteins of the invention are administered to treat, inhibit or prevent an immunological disorder. Gene therapy refers to therapy performed by the administration to a subject of an expressed or expressible nucleic acid. In this embodiment of the invention, the nucleic acids produce their encoded protein that mediates a therapeutic effect.

Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

For general reviews of the methods of gene therapy, *see*, Goldspiel *et al.*, 1993, Clinical Pharmacy 12:488-505; Wu and Wu, 1991, Biotherapy 3:87-95; Tolstoshev, 1993, Ann. Rev. Pharmacol. Toxicol. 32:573-596; Mulligan, 1993, Science 260:926-932; Morgan and Anderson, 1993, Ann. Rev. Biochem. 62:191-217; May, 1993, TIBTECH 1, l(5):155-215. Methods commonly known in the art of recombinant DNA technology which can be used are described in Ausubel *et al.* (eds.), Current Protocols in Molecular Biology, John Wiley & Sons, NY (1993); and Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990).

In a preferred aspect, the therapeutic comprises nucleic acid sequences encoding an antibody, said nucleic acid sequences being part of expression vectors that express the antibody or fragments or chimeric proteins or heavy or light chains thereof in a suitable host. In particular, such nucleic acid sequences have promoters operably linked to the antibody coding region, said promoter being inducible or constitutive, and, optionally, tissue- specific. In another particular embodiment, nucleic acid molecules are used in which the antibody coding sequences and any other desired sequences are flanked by regions that promote homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the antibody encoding nucleic acids (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438. In specific embodiments, the expressed antibody molecule is a single chain antibody; alternatively, the nucleic acid sequences include sequences encoding both the heavy and light chains, or fragments thereof, of the antibody.

Delivery of the nucleic acids into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid- carrying vectors, or indirect, in which case, cells are first transformed with the nucleic acids *in vitro*, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

In a specific embodiment, the nucleic acid sequences are directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, for example by constructing them as part of an appropriate nucleic acid expression vector and administering the vector so that the nucleic acid sequences become intracellular. Gene therapy vectors can be administered by infection using defective or attenuated retrovirals or other viral vectors (*see, e.g.*, U.S. Patent No. 4,980,286); direct injection of naked DNA; use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont); coating with lipids or cell-surface receptors or transfecting agents; encapsulation in liposomes, microparticles, or microcapsules; administration in linkage to a peptide which is known to enter the nucleus; administration in linkage to a ligand subject to receptor-mediated endocytosis (*see, e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432) (which can be used to target cell types specifically expressing the receptors); *etc.* In another embodiment, nucleic acid-ligand

complexes can be formed in which the ligand comprises a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted *in vivo* for cell specific uptake and expression, by targeting a specific receptor (*see, e.g.*, PCT Publications WO 92/06 180; WO 92/22635; W092/20316; W093/14188, and WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression by homologous recombination (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra *et al.*, 1989, Nature 342:435-438).

In a specific embodiment, viral vectors that contain nucleic acid sequences encoding an antibody of the invention are used. For example, a retroviral vector can be used (see Miller *et al.*, 1993, Meth. Enzymol. 217:581-599). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding the antibody to be used in gene therapy are cloned into one or more vectors, thereby facilitating delivery of the gene into a patient. More detail about retroviral vectors can be found in Boesen *et al.*, 1994, Biotherapy 6:29 1-302, which describes the use of a retroviral vector to deliver the mdr 1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, 1994, J. Clin. Invest. 93:644-651; Klein *et al.*, 1994, Blood 83:1467-1473; Salmons and Gunzberg, 1993, Human Gene Therapy 4:129-141; and Grossman and Wilson, 1993, Curr. Opin. in Genetics and Devel. 3:110-114.

Another approach to gene therapy involves transferring a gene, *e.g.* an AC10 or HeFi-1 gene, to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a selectable marker to the cells. The cells are then placed under selection to isolate those cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration *in vivo* of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing

the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell mediated gene transfer, spheroplast fusion, *etc.* Numerous techniques are known in the art for the introduction of foreign genes into cells (*see, e.g.*, Loeffler and Behr, 1993, Meth. Enzymol. 217:599-618; Cohen *et al.*, 1993, Meth. Enzymol. 217:618-644; Cline, 1985, Pharmac. Ther. 29:69-92) and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. Recombinant blood cells (*e.g.*, hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, *etc.*, and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to fibroblasts; blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, *e.g.*, as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, *etc.*

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, nucleic acid sequences encoding an antibody are introduced into the cells such that they are expressible by the cells or their progeny, and the recombinant cells are then administered *in vivo* for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem and/or progenitor cells which can be isolated and maintained *in vitro* can potentially be used in accordance with this embodiment of the present invention (see *e.g.* PCT Publication WO 94/08598; Stemple and Anderson, 1992, Cell 71:973-985; Rheinwald, 1980, Meth. Cell Bio. 21A:229; and Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

The compounds or pharmaceutical compositions of the invention are preferably tested *in vitro*, and then *in vivo* for the desired therapeutic or prophylactic activity, prior to use in humans. For example, *in vitro* assays to demonstrate the therapeutic or prophylactic utility of an protein or pharmaceutical composition include determining the effect of the protein or pharmaceutical composition on an activated lymphocytes. The cytotoxic and/or cytostatic effect of the protein or composition on the activated lymphocytes can be determined utilizing techniques known to those of skill in the art. Alternatively, *in vitro* assays which can be used to determine whether administration of a specific protein or pharmaceutical composition is indicated, include *in vitro* cell culture assays in which activated lymphocytes, including activated lymphocytes from a patient, are grown in culture, and exposed to or otherwise a protein or pharmaceutical composition, and the effect of such compound upon the activated lymphocytes is observed.

5.11 THERAPEUTIC/PROPHYLACTIC ADMINISTRATION AND COMPOSITIONS

The invention provides methods of treatment and prophylaxis by administration to a subject of an effective amount of a CD30-binding protein which has a cytotoxic or cytostatic effect on activated lymphocytes (*i.e.*, a protein of the invention), a nucleic acid encoding said CD30-binding protein (*i.e.*, a nucleic acid of the invention), or a pharmaceutical composition comprising a protein or nucleic acid of the invention (hereinafter, a pharmaceutical of the invention).

The outcome of the present therapeutic and prophylactic methods is to at least produce in a patient a healthful benefit, which includes but is not limited to: prolonging the lifespan of a patient, prolonging the onset of symptoms of an immune disorder, and/or alleviating a symptom of the immune disorder after onset of a symptom. Such a healthful benefit can result inhibiting disease progression and/or reducing disease symptoms.

As used herein, the term "prevention" refers to administration of a protein or nucleic acid of the invention to the patient before the onset of symptoms or molecular indications of the immune disorder of interest, for example to an individual with a predisposition or at a high risk of acquiring the immune disorder. In contrast, the term "treatment" refers to administration of a protein or nucleic acid of the present invention to the patient after the onset of symptoms or molecular indications of the immune disorder at any clinical stage.

In a preferred embodiment, the protein of the invention is the monoclonal antibody AC10 or HeFi-1 or a fragment or derivative thereof. In a preferred aspect, a pharmaceutical of the invention comprises a substantially purified protein or nucleic acid of the invention (*e.g.*, substantially free from substances that limit its effect or produce undesired side-effects).

The subject is preferably an animal, and is preferably a mammal, including but not limited to animals such as cows, pigs, horses, chickens, cats, dogs, *etc.* Most preferably, the subject is human.

Formulations and methods of administration that can be employed are described above; additional appropriate formulations and routes of administration can be selected from among those described herein below.

Various delivery systems are known and can be used to administer a nucleic acid or protein of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (*see*, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, *etc.* Methods of introduction include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural, and oral routes. Nucleic acids and proteins of the invention may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents such as chemotherapeutic agents (see Section 5.2.1). Administration can be systemic or local.

In a specific embodiment, it may be desirable to administer the nucleic acid or protein of the invention by injection, by means of a catheter, by means of a suppository, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including a membrane, such as a sialastic membrane, or a fiber. Preferably, when administering a protein, including an antibody, of the invention, care must be taken to use materials to which the protein does not absorb.

In another embodiment, the compound or composition can be delivered in a vesicle, in particular a liposome (*see* Langer, 1990, Science 249:1527-1533; Treat *et al.*, 1989, in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353- 365; Lopez-Berestein, ibid., pp. 317-327; see generally, *ibid.*)

In yet another embodiment, the compound or composition can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer, *supra*; Sefton, 1989, CRC Crit. Ref. Biomed. Eng. 14:201; Buchwald *et al.*, 1980, Surgery 88:507; Saudek *et al.*, 1989, N. Engl. J. Med. 321:574). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, 1974, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida; Controlled Drug Bioavailability, Drug Product Design and Performance, 1984, Smolen and Ball (eds.), Wiley, New York; Ranger and Peppas, 1983, Macromol. Sci. Rev. Macromol. Chem. 23:61; see also Levy *et al.*, 1985, Science 228:190; During *et al.*, 1989, Ann. Neurol. 25:351; Howard *et al.*, 1989, J. Neurosurg. 71:105).

Other controlled release systems are discussed in the review by Langer, 1990, Science 249:1527-1533.

In a specific embodiment where a nucleic acid of the invention is administered, the nucleic acid can be administered *in vivo* to promote expression of its encoded protein, by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, *e.g.*, by use of a retroviral vector (*see* U.S. Patent No. 4,980,286), or by direct injection, or by use of microparticle bombardment (*e.g.*, a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, or by administering it in linkage to a homeobox- like peptide which is known to enter the nucleus (*see e.g.*, Joliot *et al.*, 1991, Proc. Natl. Acad.

Sci. USA 88:1864-1868), *etc.* Alternatively, a nucleic acid can be introduced intracellularly and incorporated within host cell DNA for expression, by homologous recombination.

As alluded to above, the present invention also provides pharmaceutical compositions (pharmaceuticals of the invention). Such compositions comprise a therapeutically effective amount of a nucleic acid or protein of the invention, and a pharmaceutically acceptable carrier. In a specific embodiment, the term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water is a preferred carrier when the pharmaceutical composition is administered intravenously. Saline solutions and aqueous dextrose and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc.* Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin. Such compositions will contain a therapeutically effective amount of the nucleic acid or protein of the invention, preferably in purified form, together with a suitable amount of carrier so as to provide the form for proper administration to the patient. The formulation should suit the mode of administration.

In a preferred embodiment, the pharmaceutical of the invention is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the pharmaceutical of the invention may also include a solubilizing agent and a local anesthetic such as lignocaine to ease pain at the site of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a dry lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the pharmaceutical of the invention is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the pharmaceutical of the invention is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

The amount of the nucleic acid or protein of the invention which will be effective in the treatment or prevention of an immunological disorder can be determined by standard clinical techniques. In addition, *in vitro* assays may optionally be employed to help identify optimal dosage ranges. The precise dose to be employed in the formulation will also depend on the route of administration, and the stage of immunological disorder, and should be decided according to the judgment of the practitioner and each patient's circumstances. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems.

5.12 COMBINATION THERAPY FOR TREATMENT OF IMMUNOLOGICAL DISORDERS

The nucleic acids and proteins of the invention can be administered together with one or more cytostatic, cytotoxic and/or immunosuppressive agents for the treatment and prevention of immunological disorders. Additionally, combination therapy may include administration of an agent that targets a receptor or receptor complex other than CD30 on the surface of activated lymphocytes. An example of such an agent is a second, non-CD30 antibody that binds to a molecule at the surface of an activated lymphocyte. Another example is a ligand that targets such a receptor or receptor

complex. Preferably, such an antibody or ligand binds to a cell surface receptor on activated lymphocytes and enhances the cytotoxic or cytostatic effect of the anti-CD30 antibody by delivering a cytostatic or cytotoxic signal to the activated lymphocytes.

Such combinatorial administration can have an additive or synergistic effect on disease parameters.

With respect to therapeutic regimens, in a specific embodiment, a nucleic acid or protein of the invention is administered concurrently with an immunsuppressive agent or a molecule that targets a lymphocyte cell surface receptor or receptor complex. In another specific embodiment, the immunosuppressive agent or lymphocyte cell surface receptor targeting-agent is administered prior or subsequent to administration of a nucleic acid or protein of the invention, by at least an hour and up to several months, for example at least an hour, five hours, 12 hours, a day, a week, a month, or three months, prior or subsequent to administration of a nucleic acid or protein of the invention.

5.12.1 IMMUNOSUPPRESSIVE, CYTOTOXIC AND CYTOSTATIC AGENTS

A useful class of immunosuppressive, cytotoxic or cytostatic agents for practicing the combinatorial therapeutic regimens of the present invention include, but are not limited to, the following non-mutually exclusive classes of agents: alkylating agents, anthracyclines, antibiotics, antifolates, antimetabolites, antitubulin agents, auristatins, chemotherapy sensitizers, DNA minor groove binders, DNA replication inhibitors, duocarmycins, etoposides, fluorinated pyrimidines, lexitropsins, nitrosoureas, platinols, purine antimetabolites, puromycins, radiation sensitizers, steroids, taxanes, topoisomerase inhibitors, and vinca alkaloids.

Individual immunosuppressive, cytotoxic or cytostatic agents encompassed by the invention include but are not limited to an androgen, anthramycin (AMC), asparaginase, 5-azacytidine, azathioprine, bleomycin, busulfan, buthionine sulfoximine, camptothecin, carboplatin, carmustine (BSNU), CC-1065, chlorambucil, cisplatin, colchicine, cyclophosphamide, cytarabine, cytidine arabinoside, cytochalasin B, dacarbazine, dactinomycin (formerly actinomycin), daunorubicin, decarbazine, docetaxel, doxorubicin, an estrogen, 5-fluordeoxyuridine, 5-fluorouracil, gramicidin D, hydroxyurea,

idarubicin, ifosfamide, irinotecan, lomustine (CCNU), mechlorethamine, melphalan, 6-mercaptopurine, methotrexate, mithramycin, mitomycin C, mitoxantrone, nitroimidazole, paclitaxel, plicamycin, procarbizine, streptozotocin, tenoposide, 6-thioguanine, thioTEPA, topotecan, vinblastine, vincristine, vinorelbine, VP-16 and VM-26.

In a preferred embodiment, the immunosuppressive, cytotoxic or cytostatic agent is an antimetabolite. The antimetabolite can be a purine antagonist (*e.g.* azothioprine) or mycophenolate mofetil), a dihydrofolate reductase inhibitor (*e.g.*, methotrexate), acyclovir, gangcyclovir, zidovudine, vidarabine, ribavarin, azidothymidine, cytidine arabinoside, amantadine, dideoxyuridine, iododeoxyuridine, poscarnet, and trifluridine.

In another preferred embodiment, the immunosuppressive, cytotoxic or cytostatic agent is tacrolimus, cyclosporine or rapamycin.

In another preferred embodiment, the immunosuppressive agent is a glucocorticoid or glucocorticoid analogue. Examples of glucocorticoids useful in the present methods include cortisol and aldosterone. Examples of glucocorticoid analogues useful in the present methods include prednisone and dexamethasone.

In yet another preferred embodiment, the immunosuppressive agent is an anti-inflammatory agent, such as consisting arylcarboxylic derivatives, pyrazole-containing derivatives, oxicam derivatives and nicotinic acid derivatives. Classes of anti-inflammatory agents useful in the methods of the present invention include cyclooxygenase inhibitors, 5-lipoxygenase inhibitors, and leukotriene receptor antagonists.

Suitable cyclooxygenase inhibitors include meclofenamic acid, mefenamic acid, carprofen, diclofenac, diflunisal, fenbufen, fenoprofen, ibuprofen, indomethacin, ketoprofen, nabumetone, naproxen, sulindac, tenoxicam, tolmetin, and acetylsalicylic acid.

Suitable lipoxygenase inhibitors include redox inhibitors (*e.g.*, catechol butane derivatives, nordihydroguaiaretic acid (NDGA), masoprocol, phenidone, Ianopalen, indazolinones, naphazatrom, benzofuranol, alkylhydroxylamine), and non-redox inhibitors (*e.g.*, hydroxythiazoles, methoxyalkylthiazoles, benzopyrans and derivatives thereof, methoxytetrahydropyran, boswellic acids and acetylated derivatives of

boswellic acids, and quinolinemethoxyphenylacetic acids substituted with cycloalkyl radicals), and precursors of redox inhibitors.

Other suitable lipoxygenase inhibitors include antioxidants (*e.g.*, phenols, propyl gallate, flavonoids and/or naturally occurring substrates containing flavonoids, hydroxylated derivatives of the flavones, flavonol, dihydroquercetin, luteolin, galangin, orobol, derivatives of chalcone, 4,2',4'-trihydroxychalcone, ortho-aminophenols, N-hydroxyureas, benzofuranols, ebselen and species that increase the activity of the reducing selenoenzymes), iron chelating agents (*e.g.*, hydroxamic acids and derivatives thereof, N-hydroxyureas, 2-benzyl-1-naphthol, catechols, hydroxylamines, carnosol trolox C, catechol, naphthol, sulfasalazine, zyleuton, 5-hydroxyanthranilic acid and 4-(omega-arylalkyl)phenylalkanoic acids), imidazole-containing compounds (*e.g.*, ketoconazole and itraconazole), phenothiazines, and benzopyran derivatives.

Yet other suitable lipoxygenase inhibitors include inhibitors of eicosanoids (*e.g.*, octadecatetraenoic, eicosatetraenoic, docosapentaenoic, eicosahexaenoic and docosahexaenoic acids and esters thereof, PGE1 (prostaglandin E1), PGA2 (prostaglandin A2), viprostol, 15-monohydroxyeicosatetraenoic, 15-monohydroxy-eicosatrienoic and 15-monohydroxyeicosapentaenoic acids, and leukotrienes B5, C5 and D5), compounds interfering with calcium flows, phenothiazines, diphenylbutylamines, verapamil, fuscoside, curcumin, chlorogenic acid, caffeic acid, 5,8,11,14-eicosatetrayenoic acid (ETYA), hydroxyphenylretinamide, Ionapalen, esculin, diethylcarbamazine, phenantroline, baicalein, proxicromil, thioethers, diallyl sulfide and di-(1-propenyl) sulfide.

Leukotriene receptor antagonists include calcitriol, ontazolast, Bayer Bay-x-1005, Ciba-Geigy CGS-25019C, ebselen, Leo Denmark ETH-615, Lilly LY-293111, Ono ONO-4057, Terumo TMK-688, Boehringer Ingleheim BI-RM-270, Lilly LY 213024, Lilly LY 264086, Lilly LY 292728, Ono ONO LB457, Pfizer 105696, Perdue Frederick PF 10042, Rhone-Poulenc Rorer RP 66153, SmithKline Beecham SB-201146, SmithKline Beecham SB-201993, SmithKline Beecham SB-209247, Searle SC-53228, Sumitamo SM 15178, American Home Products WAY 121006, Bayer Bay-o-8276, Warner-Lambert CI-987, Warner-Lambert CI-987BPC-15LY 223982, Lilly LY 233569, Lilly LY-255283, MacroNex MNX-160, Merck and Co. MK-591, Merck and

CO. MK-886, Ono ONO-LB-448, Purdue Frederick PF-5901, Rhone-Poulenc Rorer RG 14893, Rhone-Poulenc Rorer RP 66364, Rhone-Poulenc Rorer RP 69698, Shionoogi S-2474, Searle SC-41930, Searle SC-50505, Searle SC-51146, Searle SC-52798, SmithKline Beecham SK&F-104493, Leo Denmark SR-2566, Tanabe T-757 and Teijin TEI-1338.

In certain preferred embodiments of the present invention, the immunosuppressive, cytotoxic or cytostatic agent is conjugated to an antibody of the invention rather than being administered separately. Antibody-drug conjugates useful in the present methods are described in Section 5.6, *supra*.

5.12.2 LYMPHOCYTE RECEPTOR TARGETING AGENTS

Agents that are particularly useful in the present combinatorial methods are molecules that bind to lymphocyte cell surface, preferably against a receptor or receptor complex distinct from CD30. Besides CD30, a wide variety of receptors or receptor complexes expressed on lymphocyte surface are involved in regulating the proliferation, differentiation, and functions of different lymphocyte subsets. Such molecules can be targeted, for example, to provide additional cytostatic or cytotoxic signals to activated lymphocytes.

In one embodiment, suitable receptors for targeting alongside CD30 are immunoglobulin gene superfamily members, including but not limited to CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, and ICOS (Barclay *et al.*, 1997, The Leucocyte Antigen FactsBook, 2nd ed, Academic Press; Coyle and Gurtierrez-Ramos, 2001, Nature Immunol. 2:203-209). In another embodiment, TNF receptor superfamily members can be targeted, including but not limited to CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, and APO-3. (Locksley *et al.*, 2001, Cell, 104, 487-501). In yet another embodiment, an integrin can be targeted, including but not limited to CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, and CD104 (Barclay *et al.*, 1997, The Leucocyte Antigen FactsBook, 2nd ed, Academic Press). In yet other embodiments, a suitable receptor for targeting in addition to CD30 is a cytokine receptor

(Fitzgerald *et al.*, 2001, The Cytokine Factsbook, 2nd ed, Academic Press), a chemokine receptor (Luther and Cyster, 2001, Nature Immunol. 2:102-107; Gerard and Rollins, 2001, Nature Immunol. 2: 108-115), a major histocompatibility protein, a lectin (C-type, S-type, or I-type), or a complement control protein.

In one embodiment, agents that bind to these non-CD30 receptors or receptor complexes enhance the cytotoxic or cytostatic effect of the CD30-binding agent (*e.g.*, an anti-CD30 antibody) by delivering a cytostatic or cytotoxic signal to the activated lymphocytes. In combination with the anti-CD30 molecules of the invention, an additive or synergistic effect on growth inhibition or apoptosis can be achieved in the targeted lymphocyte.

In another embodiment, agents against these receptors or receptor complexes need not be growth inhibitory or apoptotic on their own, but, in combination with a CD30-binding agent, an enhanced effect on growth inhibition or apoptosis beyond that induced by the CD30-binding agent alone can be achieved. In certain specific embodiments, the enhanced effect is approximately a 5%, 10% 15%, 20%, 25%, 30%, 40%, 50%, 75%, 100% or greater enhancement in the cytostatic or cytotoxic activity of a given amount or concentration of a CD30-binding agent. In one embodiment, the enhanced effect refers to an approximately 5%, 10% 15%, 20%, 25%, 30%, 40%, 50%, 75% reduction in the ED_{50} of the CD30-binding agent, *i.e.*, the amount of the CD30-binding agent capable of achieving the same cytotoxic or cytostatic effect is less than what would be required to achieve the same cytotoxic or cytostatic effect in the absence of administration of such agents that bind to receptor or receptor complexes other than CD30.

In one embodiment, targeting a non-CD30 receptor or receptor complex according to the methods of the present invention can be achieved by administering a ligand.

In another embodiment, targeting can be achieved by administering an antibody against the receptor or receptor complex. The antibody can be a polyclonal antibody, a monoclonal antibody, an epitope-binding antibody fragment, or another type of antibody derivative equivalent to those anti-CD30 derivatives described in Sections 5.1 and 5.4, *supra*. In certain specific embodiments, the antibody is a multivalent antibody or

a heteroconjugate comprising a CD30-binding portion, as described in Sections 5.1 and 5.4.

A number of antibodies suitable for co-administration with anti-CD30 are known in the art, as will be recognized by the skilled artisan. Listed below are exemplary, non-limiting examples of such antibodies: the anti-CD2 antibodies include BTI-322 (Medimmune) and UMCD2; the anti-CD3 antibodies OKT3, "SMART" Anti-CD3 (Nuvion™; Protein Design Laboratories), FN18, UCHT1, 145-2C11, and HIT3a; the anti-CD5 antibodies HI211 (6T-003), HISM2 (6T-004), MEM-128 (6T-014), 7.8 (6T-080, OKT1, UCHT2, and BL1a; the anti-CTLA-4 antibodies 11D4, 10A8, 7F8, 4F10, ANC152.2/8H5, and BNI3.1; and the anti-PD-1 antibody J43.

Natural ligands have also been defined for many of the receptors or receptor complexes (Barclay *et al.*, 1997, The Leucocyte Antigen FactsBook, 2nd ed, Academic Press; Coyle and Gurtierrez-Ramos, 2001, Nature Immunol. 2:203-20; Locksley *et al.*, 2001, Cell 104:487-501). Listed below are exemplary, non-limiting examples of such ligands: LFA-3, a ligand for CD2; CD80 and CD86, ligands for CD28 and CTLA-4; PD-L1 and PD-L2, ligands for PD-1; B7RP-1, a ligand for ICOS; CD70, a ligand for CD27; CD154, a ligand for CD40; FasL, a ligand for CD95/Fas; TNFa, a ligand for TNF-R1 and TNF-R2; TRANCE, a ligand for RANK, APRIL, a ligand for TACI; BLYS, a ligand for BCMA, TRAIL, a ligand for TRAIL-R1, -R2, -R3, and R4; and TWEAK, a ligand for APO-3.

5.13 EFFECTIVE DOSE

Toxicity and therapeutic efficacy of the proteins and compositions of the invention can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD_{50} (the dose lethal to 50% of the population) and the ED_{50} (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD_{50}/ED_{50}. Proteins that exhibit large therapeutic indices are preferred. While proteins that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such proteins to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such proteins lies preferably within a range of circulating concentrations that include the ED_{50} with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the IC_{50} (*i.e.*, the concentration of the test compound that achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

Generally, the dosage of a protein of the invention in a pharmaceutical of the invention administered to a immunological disorders patient is typically 0.1 mg/kg to 100 mg/kg of the patient's body weight. Preferably, the dosage administered to a patient is between 0.1 mg/kg and 20 mg/kg of the patient's body weight, more preferably 1 mg/kg to 10 mg/kg of the patient's body weight. Generally, human antibodies have a longer half-life within the human body than antibodies from other species due to the immune response to the foreign proteins. Thus, lower dosages of humanized, chimeric or human antibodies and less frequent administration is often possible.

5.14 FORMULATIONS

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically acceptable carriers or excipients. Pharmaceutical compositions comprising the anti-CD30 antibodies of the invention may further comprise a second antibody, such as an antibody described in Section 5.12.2, *supra*, or an immunosuppressive agent, such as one of those enumerated in Section 5.12.1, *supra*.

Thus, the proteins and their physiologically acceptable salts and solvates may be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal, parenteral or rectal administration.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (*e.g.*, pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (*e.g.*, lactose, microcrystalline cellulose or calcium hydrogen phosphate) lubricants (*e.g.*, magnesium stearate, talc or silica); disintegrants (*e.g.*, potato starch or sodium starch glycolate); or wetting agents (*e.g.*, sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicles before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (*e.g.*, sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (*e.g.*, lecithin or acacia); non-aqueous vehicles (*e.g.*, almond oil, oily esters, ethyl alcohol or fractionated vegetable oils); and preservatives (*e.g.*, methyl or propyl-p-hydroxybenzoates or sorbic acid). The preparations may also contain buffer salts, flavoring, coloring and sweetening agents as appropriate.

Preparations for oral administration may be suitably formulated to give controlled release of the active compound.

For buccal administration the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the proteins for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The proteins may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, *e.g.*, in ampoules or in multidose containers, with an added

preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The proteins may also be formulated in rectal compositions such as suppositories or retention enemas, *e.g.*, containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the proteins may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the proteins may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The compositions may, if desired, be presented in a pack or dispenser device that may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration preferably for administration to a human.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

The invention is further described in the following examples which are in no way intended to limit the scope of the invention.

6. EXAMPLE 1: EXPRESSION OF CD30 ON THE JURKAT HUMAN T LYMPHOCYTE CELL LINE

The Jurkat cell line is an acute T leukemia cell line that expresses the CD3/T cell receptor (TCR) complex and other important accessory molecules involved in T cell functions. Sub-lines derived from the original Jurkat line have been applied extensively as model systems to elucidate signaling pathways mediated by a multitude of receptor systems, e.g., CD3/T cell receptor (TCR). A number of signaling pathways in Jurkat T cells initiated upon the ligation of surface receptors have been demonstrated to take place in normal T lymphocytes subjected to antigenic challenge. Jurkat T cells were found to consistently express detectable levels of CD30 (FIG. 1), and therefore they may be a model system to examine the function of CD30 in activated lymphocytes.

7. EXAMPLE 2: CROSS-LINKING OF CD30 ON JURKAT T CELLS BY ANTI-CD30 MABS INHIBITED DNA SYNTHESIS

The effect of signaling through CD30 by anti-CD30 on the proliferation of Jurkat T cells was assessed by tritiated thymidine (^{3}H-TdR) incorporation assays. Jurkat cells were treated with soluble anti-CD30 in graded doses or anti-CD30 cross-linked by a secondary cross-linking antibody (Ab). For secondary cross-linking of cAC10, the monoclonal antibody (mAb) was mixed with $F(ab')_2$ fragments of goat anti-human (GAM) IgG Fc (Jackson ImmunoResearch, West Groove, PA) in culture medium (RPMI-1640, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids). For secondary cross-linking of murine anti-CD30 mAb (AC10 and HeFi-1), the mAbs were mixed with $F(ab')_2$ fragments of goat anti-mouse (GAM) IgG Fc (Jackson ImmunoResearch) in culture medium. Final ratios of 1:2.5, 1:5, and 1:10 between the primary mAbs and secondary cross-linking antibodies were used. Antibody cocktails were allowed to incubate at room temperature for 15 minutes. Serial dilutions of these antibody cocktails in culture medium were then prepared to achieve the desired final concentrations. One hundred µl of antibody cocktails were then mixed with 100 µl Jurkat cell suspension containing 5000 cells in 96-well tissue culture (TC) plates. Cells were incubated with the antibody cocktails for 44 hours before a 4-hour pulse with 1 µCi of ^{3}H-TdR. Cells were then harvested onto filters, and incorporated tritiated

thymidine was measured by scintillation counting. Thymidine incorporation values obtained from the treated cells were then compared to the untreated control cultures.

Chimeric AC10 (cAC10) without secondary cross-linking did not inhibit Jurkat cell proliferation at concentrations below 1 μg/ml. Cross-linking cAC10 with a secondary Ab lowered the concentration of cAC10 needed to significantly inhibit Jurkat cell proliferation. Maximal inhibition was achieved at concentrations of cAC10 as little as 0.1 μg/ml (FIG. 2). Both AC10 and HeFi-1 inhibited thymidine incorporation in Jurkat cell dose-dependently when a secondary cross-linking antibodies was used (FIG. 3). The use of cross-linking antibodies *in vitro* likely simulates cross-linking via binding of the Fc portion of antibody to Fc receptors expressed on monocyte, macrophages, B lymphocytes, and NK cells *in vivo*. A ratio of 1:10 between the primary and secondary cross-linking antibody appeared to be most efficient for proliferation inhibition. Detectable inhibition was observed at primary antibody concentrations of more than 0.001 μg/ml for both AC10 and HeFi-1. Maximal level of inhibition was observed at concentrations greater than 0.1 μg/ml. AC10 and HeFi-1 immobilized onto TC wells were also active in inhibiting Jurkat T cell proliferation (data not shown).

8. EXAMPLE 3: CROSS-LINKING OF CD30 BY ANTI-CD30 MABS INDUCED DNA FRAGMENTATION (APOPTOSIS) IN JURKAT T CELLS

The relationship between cell cycle status and DNA replication in Jurkat cells treated with cross-linked anti-CD30 mAbs was further investigated. Four μg/ml of the anti-CD30 mAb AC10 or HeFi-1 were mixed with $F(ab')_2$ fragments of GAM IgG Fc (Jackson ImmunoResearch) at final ratios (weight:weight) of 1:4 in culture medium (RPMI-1640, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids). The antibody cocktails were allowed to incubate at room temperature for 15 minutes. Serial 1:10 dilutions of these antibody cocktails in culture medium were then prepared. One ml of antibody cocktails was then mixed with 1 ml of Jurkat cell suspension containing $0.25x10^6$ cells in 12-well TC plates.

After 24 or 48 hours of incubation, cells were harvested by centrifugation and resuspended in 2 ml of fresh medium equilibrated at 37°C. Bromodeoxyuridine

(BrdU) (Sigma, St. Louis, MO) was used to label cells that were actively synthesizing DNA. Twenty µl of 1 mM BrdU dissolved in water were added to the cell suspension, and the incubation continued at 37°C for 15 minutes. Cells were then spun down, washed once with PBS, and resuspended in 0.5 ml of ice-cold PBS. One ml of 100% ethanol at -20°C was added drop-wise to the cell suspension for cell fixation. After fixation at 4 C for 30 minutes, fixed cells were spun down. The cell pellets were resuspended in 1 ml of 2 M HCl with 0.5% Triton X-100 (Sigma) and incubated for 30 minutes at room temperature before cells were spun down. One ml of 0.1 M $Na_2B_4O_7$ at pH 8.5 was used to resuspend the cell pellets and to neutralize the acid for 10 minutes at room temperature. Cells were then pelleted by centrifugation and washed once with PBS containing 0.5% Tween 20 (Sigma) and 1% BSA. Fifty µl of PBS, 0.5% Tween 20, 1% BSA were used to resuspend each cell pellet, to which 25 µl of FITC labeled anti-BrdU (BD Immunocytometry Systems, San Jose, CA) were added, and the cell suspension were incubated at room temperature for 30 minutes. Cells were then washed twice with PBS, 0.5% Tween 20, 1% BSA, and then resuspended in 0.5 ml of PBS containing 5 µg/ml of propidium iodide (PI) (Sigma) to quantify DNA contents in cells. DNA synthesis and cell cycle status were then examined by flow cytometry.

FIG. 4 shows the appearance of Jurkat cells with DNA fragmentation in cultures treated with either AC10 or HeFi-1 cross-linked by a goat anti-mouse IgG Fc antibody. Cells with DNA fragmentation are represented by events showing less than G_0/G_1 phase DNA content (Window 1 in FIG. 4) and events showing less than G_2/M phase DNA content that did not incorporate BrdU, i.e., cells not actively synthesizing DNA, (Window 4 in FIG. 4). DNA fragmentation was detectable after 24 and 48 hours of incubation. Such DNA fragmentation in Jurkat cell is characteristics of apoptosis. These data suggest that the inhibition of proliferation of Jurkat cells shown in FIG. 5 was accompanied by cells undergoing apoptosis in response to CD30 signaling. When the percentages of cells in different parts of the cell cycle from cultures treated with graded doses of AC10 and HeFi-1 were compiled, it was evident that AC10 or HeFi-1 at concentrations of >0.002 µg/ml were able to induce substantial apoptosis in Jurkat cells, especially after 48 hours of treatment (FIG. 5). The appearance of apoptotic cells was

paralleled by a corresponding decrease in the percentages of cells in the G_0/G_1, S, and G_2/M phases of the cell cycle (FIG. 5).

9. EXAMPLE 4: CO-CROSS-LINKING OF CD3 WITH CD30 ENHANCED APOPTOSIS INDUCED IN JURKAT T CELLS

Activation-induced cell death (ACID) is a key mechanism used by the immune system to eliminate auto-reactive lymphocytes and effector lymphocytes thereby conferring tolerance to self-antigens and terminating immune responses, respectively. Ligation of CD3/TCR complexes on T lymphocytes or the sIg/B cell antigen receptor complexes on B lymphocytes activates T and B lymphocytes. Depending on the context of antigen receptor ligation and signaling through accessory receptors, lymphocytes can proliferate and differentiate into effector cells or they can undergo apoptosis. A number of accessory receptors, *e.g.*, CD19, CD27, CD28, CD40, CD134/OX40, CD137/4-1BB, and ICOS co-stimulate with the antigen receptors on B or T lymphocytes to promote cellular proliferation and/or differentiation. Hence, they are important for promoting immune responses. On the other hand, signaling through accessory receptors including CD5, CD22, CD152/CTLA4, and PD-1 inhibits lymphocyte proliferation and various lymphocyte functions. Therefore, these receptors may play important roles in the elimination of auto-reactive lymphocytes. They are also involved in the termination of immune responses by suppressing the activities of effector lymphocytes. Defects in regulating signal transduction pathways mediated by accessory receptors may contribute to autoimmune, allergic, and inflammatory diseases.

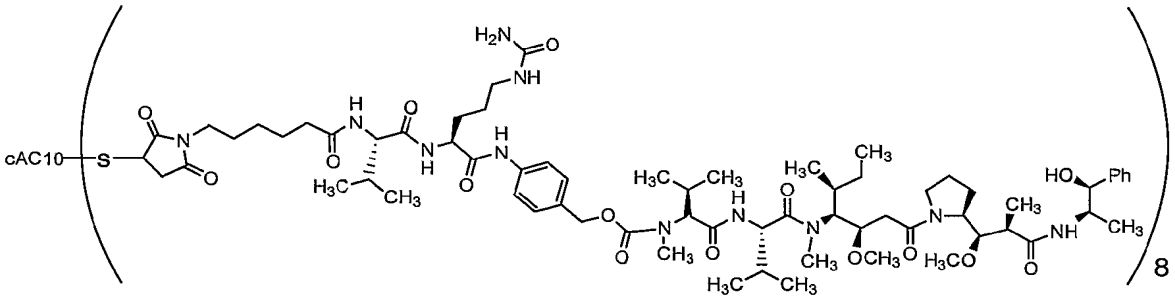
We asked the question if the CD30 signaling pathway cooperates with that of the CD3/TCR complex to regulate apoptosis in lymphocytes. Jurkat T cells were treated with either anti-CD30 mAb alone, anti-CD3 mAb, alone, or a combination of anti-CD30 and anti-CD3 mAbs. Antibodies were cross-linked by goat anti-mouse IgG Fc as described for FIG. 4 and FIG. 5. Cell cycle distribution was determined by BrdU and PI staining as in FIG. 4. Anti-CD30 (AC10) alone induced apoptosis in Jurkat T cells, whereas anti-CD3 (OKT3, ATCC, Manassas, VA) did not have any effect compared to medium control. However, anti-CD3 greatly enhanced apoptosis induced by anti-CD30

after 24 and 48 hours of incubation. The best synergistic effect between AC10 and OKT3 was observed when both mAbs were used at 0.2 μg/ml (FIG. 6).

Binding of Annexin V onto the extracellular face of the plasma membrane coupled with membrane permeability to PI was used as a complementary approach to study anti-CD30-induced apoptosis in Jurkat cells. Antibody cocktails containing AC10, HeFi-1, OKT3, AC10 + OKT3, or HeFi-1 + OKT3 cross-linked with a GAM antibody were prepared as described in Examples 2 and 3. Primary mAbs were used at a final concentration of 2 μg/ml. A 10-fold excess of GAM antibody was used to cross-link the primary antibodies. After an incubation of 15 minutes at room temperature, antibody cocktails were added to Jurkat cells. At the times designated in FIG. 7, percentages of apoptotic and dead cells in the cultures were determined by Annexin V binding and permeability to PI using the Annexin V-FITC Apoptosis Detection Kit I (BD PharMingen, San Diego, CA) according to the manufacturer's instruction. Cells that were Annexin V^-/PI^- were live cells (lower left quadrants in FIG. 7), cells that were Annexin V^+/PI^- were apoptotic cells (lower right quadrants in FIG. 7), and cells that were Annexin V^+/PI^+ were dead cells (upper right quadrants in FIG. 7). Consistent to the data obtained from PI/BrdU staining shown in FIG. 6 AC10 and HeFi-1 induced detectable apoptosis in Jurkat cells as early as 4 hours after incubation. The presence of an anti-CD3 mAb significantly enhanced the effects of AC10 and HeFi-1. The percentages of apoptotic and dead cells continued to increase during the 48 hours of incubation. These data suggest that the specific combination of anti-CD3 and anti-CD30 mAbs may have significant ability to delete CD30 positive T lymphocytes for controlled suppression of the immune responses in autoimmunity, allergic reactions and chronic inflammatory diseases.

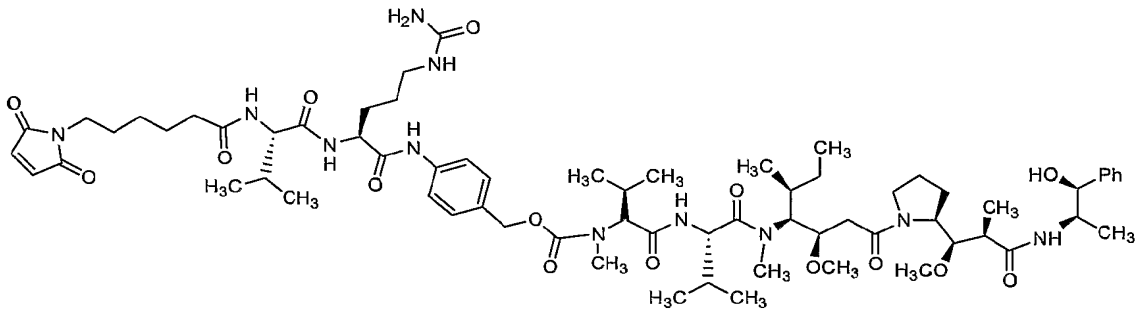
10. EXAMPLE 5: cAC10 ANTIBODY DRUG CONJUGATES (ADCs)

10.1 SYNTHESIS OF cAC10-vcMMAE



The synthesis of the ADC cAC10-vcMMAE, the general structure of which is depicted above, is described below.

10.1.1 DRUG-LINKER COMPOUND SYNTHESIS



The synthesis of auristatin E has been previously described (Pettit GR, and Barkoczy, J., 1997, US patent 5,635,483, Pettit, GR, The Dolastatins, Prog. Chem. Org. Nat. Prod., 70, 1-79, 199). The monomethyl derivative of Auristatin E (MMAE) was prepared by replacing a protected form of monomethylvaline for N,N-dimethylvaline in the synthesis of auristatin E (Senter *et al.*, U.S. provisional application no. 60/400,403 filed July 31, 2002, which is incorporated by reference herein in its entirety).

To prepare drug-linker compound, MMAE (1.69 g, 2.35 mmol), maleimidocaproyl-L-valine-L-citrulline-p-aminobenzyl alcohol p-nitrophenylcarbonate (2.6 g, 3.52 mmol, 1.5 eq., prepared as described in Dubowchik, G.M., et al., *Bioconjugate Chem.* 2002, 13, 855-869) and HOBt (64 mg, 0.45 mmol, 0.2 eq.) were diluted with DMF (25 mL). After 2 min, pyridine (5 mL) was added and the reaction was monitored using reverse-phase HPLC. The reaction was shown to be complete in 24 h. The reaction mixture was concentrated to provide a dark oil, which was diluted with 3 mL of DMF. The DMF solution was purified using flash column chromatography (silica gel, eluant gradient:100% dichloromethane to 4:1 dichloromethane-MeOH). The relevant fractions were combined and concentrated to provide an oil that solidified under high vacuum to provide a mixture of the desired drug-linker compound and unreacted MMAE as a dirty yellow solid (R_f 0.40 in 9:1 dichloromethane-MeOH). The dirty yellow solid was diluted with DMF and purified using reverse-phase preparative-HPLC (Varian Dynamax C_{18} column 41.4 mm x 25 cm, 8 μ, 100 Å, using a gradient run of MeCN and 0.1% aqueous TFA at 45 mL/min from 10% to 100% over 40 min followed by 100% MeCN for 20 min) to provide the desired drug-linker compound as an amorphous white powder (Rf 0.40 in 9:1 dichloromethane-MeOH) which was >95% pure by HPLC and which contained less than 1% of MMAE. Yield: 1.78 g (57%); ES-MS *m/z* 1316.7 $[M+H]^+$; UV λ_{max} 215, 248 nm.

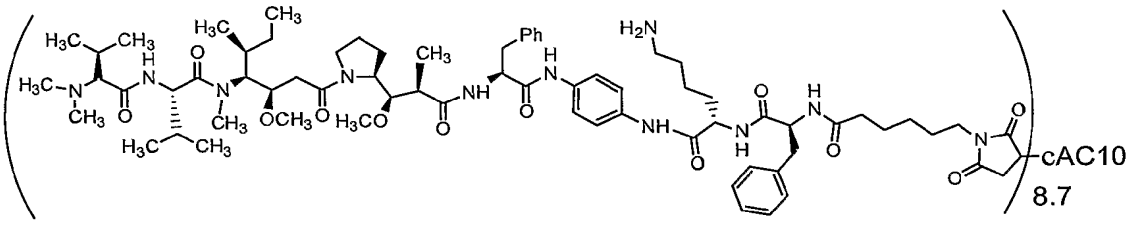
10.1.2 CONJUGATE PREPARATION

Antibody Reduction. To 4.8 mL cAC10 (10 mg/mL) was added 600 uL of 500 mM sodium borate/500 mM NaCl, pH 8.0, followed by 600 uL of 100 mM DTT in water. After incubation at 37°C for 30 min, the buffer was exchanged by elution over G25 resin equilibrated and eluted with PBS containing 1 mM DTPA (Aldrich). The thiol/Ab value was checked by determining the reduced antibody concentration from the solution's 280 nm absorbance, and the thiol concentration by reaction with DTNB (Aldrich) and determination of the absorbance at 412 nm.

Conjugation of the Reduced Antibody. The reduced mAb was chilled on ice. The drug-linker compound was used as a DMSO solution of known concentration, and the quantity of drug-linker added to the reaction mixture was calculated as follows:

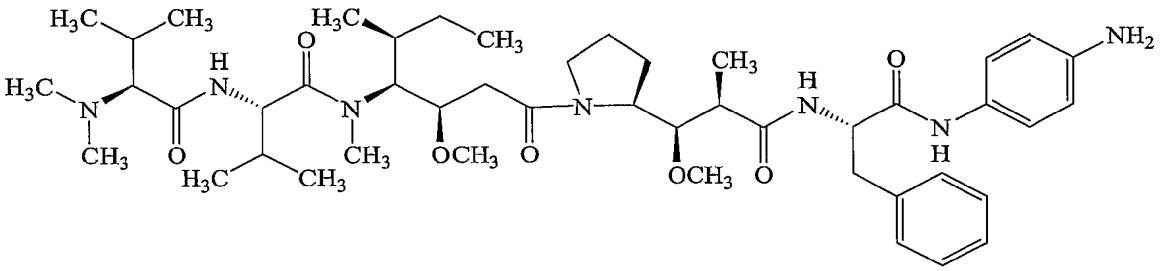
L stock solution = V x [Ab] x Fold Excess/[Drug-Linker], where V and [Ab] are the volume and molar concentration of the reduced antibody solution, respectively. 2.3 mL cold PBS/DTPA was added to the reduced antibody solution. 133.6 uL of 7.5 mM drug-linker compound stock solution was diluted into 1.47 mL acetonitrile. The acetonitrile drug-linker solution was chilled on ice, then added to the reduced antibody solution. The reaction was terminated after 1 hr by the addition of a 20 fold molar excess of cysteine over maleimide. The reaction mixture was concentrated by centrifugal ultrafiltration and purified by elution through de-salting G25 in PBS. cAC10-vcMMAE was then filtered through 0.2 micron filters under sterile conditions and immediately frozen at -80C. cAC10-vcMMAE was analyzed for 1) concentration, by UV absorbance; 2) aggregation, by size exclusion chromatography; 3) drug/Ab, by measuring unreacted thiols with DTNB, and 4) residual free drug, by reverse phase HPLC.

10.2 SYNTHESIS OF cAC10-fkAEFP



The synthesis of the ADC cAC10-fkAEFP, the general structure of which is depicted above, is described below.

10.2.1 AEFP SYNTHESIS



Boc-phenylalanine (1.0 g, 3.8 mmol) was added to a suspension of 1,4-diaminobenzene•HCl (3.5 g, 19.0 mmol, 5.0 eq.) in triethylamine (10.7 mL, 76.0 mmol, 20 eq.) and dichloromethane (50 mL). To the resulting solution was added DEPC (3.2 mL, 19.0 mmol, 5.0 eq.) via syringe. HPLC showed no remaining Boc-phe after 24 h. The reaction mixture was filtered, and the filtrate was concentrated to provide a dark solid. The dark solid residue was partitioned between 1:1 EtOAc-water, and the EtOAc layer was washed sequentially with water and brine. The EtOAc layer was dried and concentrated to provide a dark brown/red residue that was purified using HPLC (Varian Dynamax column 41.4mm x 25 cm, 5 μ, 100 Å, using a gradient run of MeCN and water at 45 mL/min form 10% to 100% over 40 min followed by 100% MeCN for 20 min). The relevant fractions were combined and concentrated to provide a red-tan solid intermediate. Yield: 1.4 g (100%); ES-MS *m/z* 355.9 $[M+H]^+$; UV λ_{max} 215, 265 nm; ^1H NMR ($CDCl_3$) δ 7.48 (1 H, br s), 7.22-7.37 (5 H, m), 7.12 (2 H, d, *J*=8.7 Hz), 7.61 (2 H, d, *J*=8.7 Hz), 5.19 (1 H, br s), 4.39-4.48 (1 H, m), 3.49 (2 H, s), 3.13 (2 H, d, *J*=5.7 Hz), 1.43 (9 H, s).

The red-tan solid intermediate (0.5 g, 1.41 mmol) and diisopropylethylamine (0.37 mL, 2.11 mmol, 1.5 eq.) were diluted with dichloromethane (10 mL), and to the resulting solution was added Fmoc-Cl (0.38 g, 1.41 mmol). The reaction was allowed to stir, and a white solid precipitate formed after a few minutes. Reaction was complete according to HPLC after 1h. The reaction mixture was filtered,

and the filtrate was concentrated to provide an oil. The oil was precipitated with EtOAc, resulting in a reddish-white intermediate product, which was collected by filtration and dried under vacuum. Yield: 0.75 g (93%); ES-MS *m/z* 578.1 $[M+H]^+$, 595.6 $[M+NH_4]^+$.

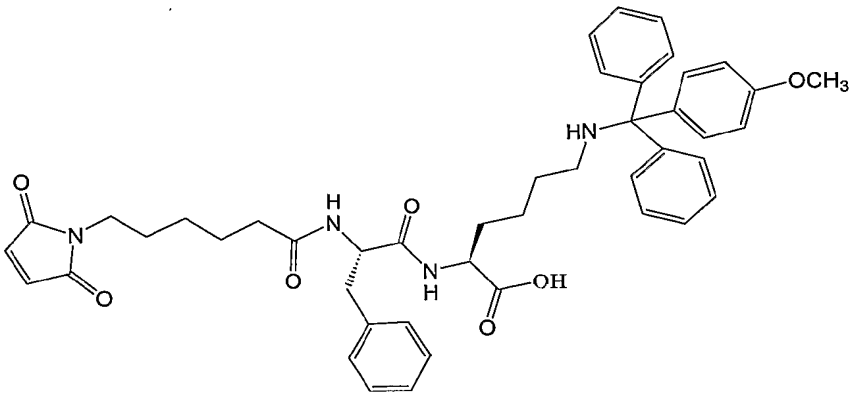
The reddish-white intermediate (0.49 g, 0.85 mmol), was diluted with 10 mL of dichloromethane, and then treated with 5 mL of trifluoroacetic acid. Reaction was complete in 30 min according to reverse-phase HPLC. The reaction mixture was concentrated and the resulting residue was precipitated with ether to provide an off-white solid. The off-white solid was filtered and dried to provide an amorphous powder, which was added to a solution of Boc-Dolaproine (prepared as described in Tetrahedron, 1993, 49(9):1913-1924) (0.24 g, 0.85 mmol) in dichloromethane (10 mL). To this solution was added triethylamine (0.36 mL, 2.5 mmol, 3.0 eq.) and PyBrop (0.59 g, 1.3 mmol, 1.5 eq.). The reaction mixture was monitored using reverse-phase HPLC. Upon completion, the reaction mixture was concentrated, and the resulting residue was diluted with EtOAc, and sequentially washed with 10% aqueous citric acid, water, saturated aqueous sodium bicarbonate, water, and brine. The EtOAc layer was dried ($MgSO_4$), filtered, and concentrated. The resulting residue was purified using flash column chromatography (silica gel) to provide an off-white powdered intermediate. Yield: 0.57 g (88%); ES-MS *m/z* 764.7 $[M+NH_4]^+$; UV λ_{max} 215, 265 nm; ^{1}H NMR (DMSO-d_3) δ 10.0-10.15 (1 H, m), 9.63 (1 H, br s), 8.42 (1/2 H, d, *J*=8.4 Hz), 8.22 (1/2 H, d, *J*=8.4 Hz), 7.89 (2 H, d, *J*=7.2 Hz), 7.73 (2 H, d, *J*=7.6 Hz), 7.11-7.55 (13 H, m), 4.69-4.75 (1 H, m), 4.46 (2 H, d, *J*=6.8 Hz), 4.29 (1 H, t, *J*=6.4 Hz), 3.29 (3 H, s), 2.77-3.47 (7 H, m), 2.48-2.50 (3 H, m), 2.25 (2/3 H, dd, *J*=9.6, 7.2 Hz), 1.41-1.96 (4 H, m), 1.36 (9 H, s), 1.07 (1 H, d, *J*=6.4 Hz, rotational isomer), 1.00 (1 H, d, *J*=6.4 Hz, rotational isomer).

The white solid intermediate (85mg, 0.11 mmol) and Me-val-val-dil-O-t-butyl (55 mg, 0.11 mmol, prepared as described in Pettit *et al. J. Chem. Soc. Perk. I*, 1996, 859) were diluted with dichloromethane (5 mL), and then treated with 2.5 mL of trifluoroacetic acid under a nitrogen atmosphere for two hours at room temperature. The reaction completion was confirmed by RP-HPLC. The solvent was removed in vacuo and the resulting residue was azeotropically dried twice with toluene, then dried under high vacuum for 12 hours.

The residue was diluted with dichloromethane (2 mL), diisopropylethylamine (3 eq.) was added, followed by DEPC (1.2 eq.). After the reaction was completed, the reaction mixture was concentrated under reduced pressure, the resulting residue was diluted with EtOAc, and washed sequentially with 10 % aqueous citric acid, water, saturated aqueous sodium bicarbonate, and brine. The EtOAc layer was dried, filtered and concentrated to provide a yellow oil.

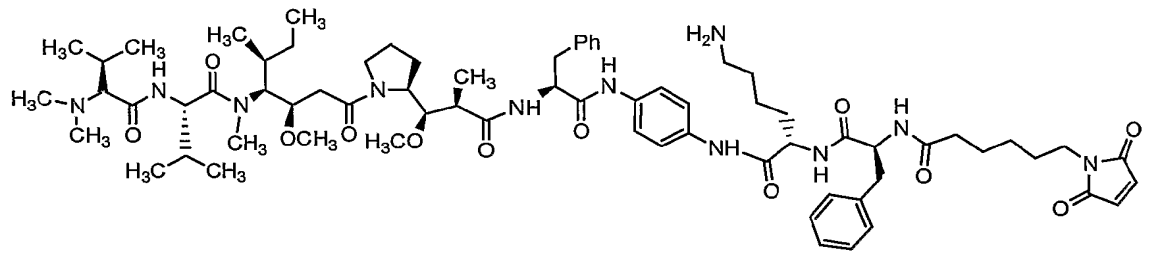
The yellow oil was diluted with dichloromethane (10 mL) and to the resulting solution diethylamine (5 mL) was added." According to HPLC, reaction was completed after 2 h. The reaction mixture was concentrated to provide an oil. The oil was diluted with DMSO, and the DMSO solution was purified using reverse phase preparative-HPLC (Varian Dynamax column 21.4 mm x 25 cm, 5 μ, 100 Å, using a gradient run of MeCN and 0.1% TFA at 20 mL/min from 10% to 100% over 40 min followed by 100% MeCN for 20 min). The relevant fractions were combined and concentrated to provide the desired drug as an off-white solid. Overall yield: 42 mg (44% overall); ES-MS *m/z* 837.8 $[M+H]^+$, 858.5 $[M+Na]^+$;UV λ_{max} 215, 248 nm.

10.2.2 LINKER SYNTHESIS



The linker compound maleimidocaproyl-L-phenylalanine-L-lysine(MMT) was prepared as described in Dubowchik *et al.*, 2002, Bioconjugate Chem. 13:855-896.

10.2.3 PREPARATION OF DRUG-LINKER COMPOUND



The drug of Section 10.2.1 (9 mg, 10.8 μmol) and the linker from section 10.2.2 (5.2 mg, 10.8 μmol) were diluted with dichloromethane (1 mL) and to the resulting solution was added HATU (6.3 mg, 16.1 μmol, 1.5 eq.), followed by pyridine (1.3 μL, 16.1 μmol, 1.5 eq.). The reaction mixture was allowed to stir under argon atmosphere while being monitored using HPLC. The reaction was complete after 6 h. The reaction mixture was concentrated and the resulting residue was diluted with DMSO. The DMSO solution was purified using reverse phase preparative HPLC (Varian Dynamax column 21.4 mm x 25 mm, 5 m, 100 Å, using a gradient run of MeCN and Et3N-CO2 (pH 7) at 20 mL/min from 10 % to 100 % over 40 min followed by 100 % MeCN for 20 min) and the relevant fractions were combined and concentrated to provide an off-white solid intermediate which was >95% pure according to HPLC.

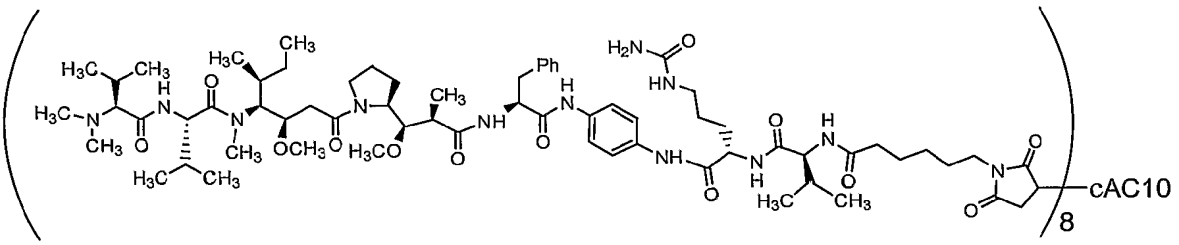
The off-white solid intermediate was diluted with dichloromethane (2 mL) and the resulting solution was treated with TFA (0.5 mL). The reaction was monitored using HPLC, and was complete in 2 h. The reaction mixture was concentrated, and the resulting residue was diluted with DMSO and purified under the same conditions as described in Example 13. The relevant fractions were combined and concentrated to provide the desired drug-linker compound as an off-white powder. Yield: 14.9 mg (90%); ES-MS *m/z* 1304.6 $[M+H]^+$; UV λ_{max} 215, 275 nm.

10.2.4 CONJUGATE PREPARATION

Antibody Reduction. To 3.0 mL cAC10 (10mg/mL) was added 375 uL of 500 mM sodium borate/500mM NaCl, pH 8.0, followed by 375 uL of 100 mM DTT in water. After incubation at 37°C for 30 min., the buffer was exchanged by elution over G25 resin equilibrated and eluted with PBS containing 1mM DTPA (Aldrich). The thiol/Ab value was checked by determining the reduced antibody concentration from the solution's 280 nm absorbance, and the thiol concentration by reaction with DTNB(Aldrich) and determination of the absorbance at 412 nm.

Conjugation of the Reduced Antibody. The reduced mAb was chilled on ice. The drug-linker compound was used as a DMSO solution of known concentration, and the quantity of drug-linker added to the reaction mixture was calculated as follows: L stock solution = V x [Ab] x Fold Excess/[Drug-Linker], where V and [Ab] are the volume and molar concentration of the reduced antibody solution, respectively. 2.2 mL cold PBS/DTPA was added to the reduced antibody solution, followed by 1.47 mL DMSO, and the mixture chilled on ice. 140.0uL of 7.6mM drug-linker compound stock solution was then added to the reduced antibody/ DMSO solution. The reaction was terminated after 1 hr by the addition of a 20 fold molar excess of cysteine over maleimide. The reaction mixture was concentrated by centrifugal ultrafiltration and purified by elution through de-salting G25 in PBS. cAC10-fkAEFP was then filtered through 0.2 micron filters under sterile conditions and immediately frozen at -80°C. cAC10-fkAEFP was analyzed for 1) concentration, by UV absorbance; 2) aggregation, by size exclusion chromatography; 3) drug/Ab, by measuring unreacted thiols by treatment with DTT, followed by DTNB, and 4) residual free drug, by reverse phase HPLC.

10.3 SYNTHESIS OF cAC10-vcAEFP:

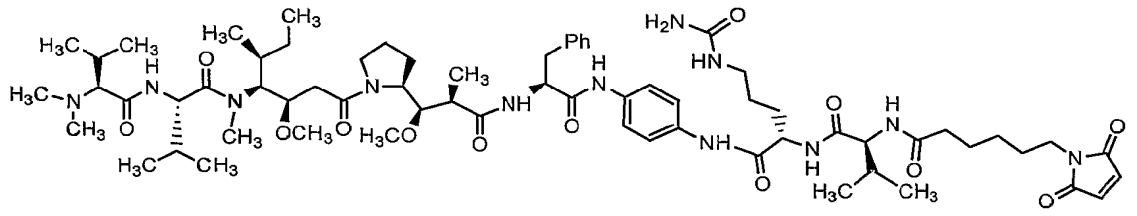


The synthesis of the ADC cAC10-vcAEFP, the general structure of which is depicted above, is described below.

10.3.1 DRUG SYNTHESIS

The drug was synthesized as illustrated above herein at Section 10.2.1.

10.3.2 PREPARATION OF DRUG-LINKER COMPOUND



The trifluoroacetate salt of the drug of Section 10.2.1 (0.37 g, 0.39 mmol, 1.0 eq.) and Fmoc-val-cit (0.30 g, 0.58 mmol, 1.5 eq., prepared according to Dubowchik et al., *Bioconjugate Chem.* 2002, 13, 855-896) were diluted with DMF (5 mL, 0.1 M), and to the resulting solution was added pyridine (95 µL, 1.2 mmol, 3.0 eq.). HATU (0.23 g, 0.58 mmol, 1.5 eq.) was then added as a solid and the reaction mixture was allowed to stir under argon atmosphere while being monitored using HPLC. The reaction progressed slowly, and 4 h later, 1.0 eq. of diisopropylethylamine was added. Reaction was complete in 1 h. The reaction mixture was concentrated *in vacuo* and the resulting residue was

purified using prep-HPLC (Varian Dynamax C18 column 41.4 mm x 25 cm, 5 μ, 100 Å, using a gradient run of MeCN and 0.1% aqueous TFA at 45 mL/min from 10% to 100% over 40 min followed by 100% MeCN for 20 min) to provide a faint pink solid intermediate.

The pink solid intermediate was diluted with DMF (30 mL) and to the resulting solution was added diethylamine (15 mL). Reaction was complete by HPLC in 2 h. The reaction mixture was concentrated and the resulting residue was washed twice with ether. The solid intermediate was dried under high vacuum and then used directly in the next step.

The solid intermediate was diluted with DMF (20 mL) and to the resulting solution was added 6-(2,5-dioxy-2,5-dihydro-pyrrol-1-yl)-hexanoic acid 2,5-dioxy-pyrrolidin-1-yl ester (0.12 g, 0.39 mmol, 1.0 eq.) (EMCS, Molecular Biosciences Inc., Boulder, CO). After 4 d, the reaction mixture was concentrated to provide an oil which was purified using prep-HPLC (Varian Dynamax C18 column 41.4 mm x 25 cm, 5 μ, 100 Å, using a gradient run of MeCN and 0.1% aqueous TFA at 45 mL/min from 10% to 100% over 40 min followed by 100% MeCN for 20 min) to provide the desired drug-linker compound as a white flaky solid. Yield: 0.21 g (38% overall); ES-MS m/z 1285.9 [M+H]+; 13.07.8 [M+Na]+; UV λ_{max} 215, 266 nm.

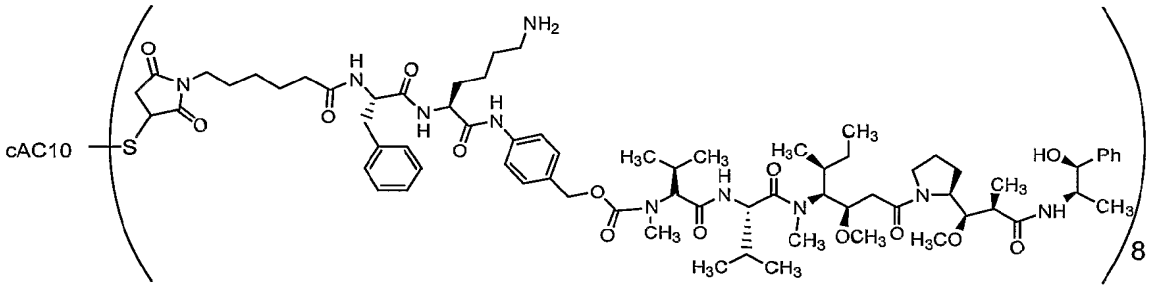
10.3.3 CONJUGATE PREPARATION

Antibody Reduction. To 3.0 mL cAC10 (10mg/mL) was added 375 uL of 500 mM sodium borate/500mM NaCl, pH 8.0, followed by 375 uL of 100 mM DTT in water. After incubation at 37°C for 30 min., the buffer was exchanged by elution over G25 resin equilibrated and eluted with PBS containing 1mM DTPA (Aldrich). The thiol/Ab value was checked by determining the reduced antibody concentration from the solution's 280 nm absorbance, and the thiol concentration by reaction with DTNB(Aldrich) and determination of the absorbance at 412 nm.

Conjugation of the Reduced Antibody. The reduced mAb was chilled on ice. The drug-linker compound was used as a DMSO solution of known concentration, and the quantity of drug-linker added to the reaction mixture was calculated as follows: L stock solution = V x [Ab] x Fold Excess/[Drug-Linker], where V and [Ab] are the volume

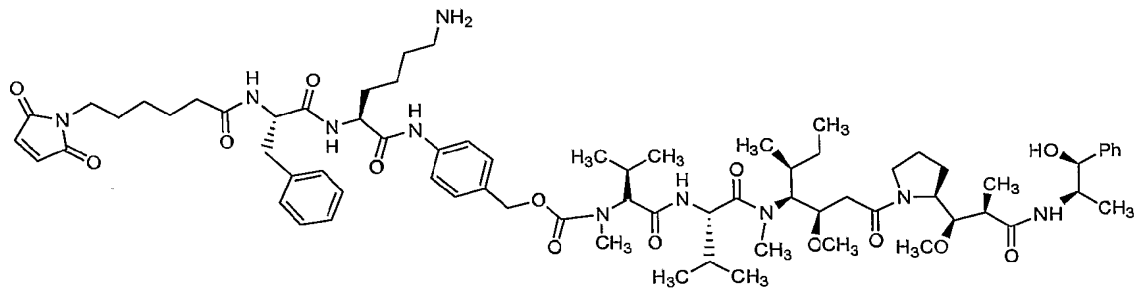
and molar concentration of the reduced antibody solution, respectively. 2.2 mL cold PBS/DTPA was added to the reduced antibody solution, followed by 1.47 mL DMSO, and the mixture chilled on ice. 140.0uL of 7.6mM drug-linker compound stock solution was then added to the reduced antibody/DMSO solution. The reaction was terminated after 1 hr by the addition of a 20 fold molar excess of cysteine over maleimide. The reaction mixture was concentrated by centrifugal ultrafiltration and purified by elution through de-salting G25 in PBS. cAC10-vcAEFP was then filtered through 0.2 micron filters under sterile conditions and immediately frozen at -80°C. cAC10-vcAEFP was analyzed for 1) concentration, by UV absorbance; 2) aggregation, by size exclusion chromatography; 3) drug/Ab, by measuring unreacted thiols by treatment with DTT, followed by DTNB, and 4) residual free drug, by reverse phase HPLC.

10.4 SYNTHESIS OF cAC10-fkMMAE



The synthesis of the ADC cAC10-fkMMAE , the general structure of which is depicted above, is described below.

10.4.1 PREPARATION OF DRUG-LINKER COMPOUND



The synthesis of auristatin E has been previously described (Pettit GR, and Barkoczy, J., 1997, U.S. patent 5,635,483, Pettit, G.R., *Prog. Chem. Org. Nat. Prod.*, 70, 1-79, 199). The monomethyl derivative of Auristatin E (MMAE) was prepared by replacing a protected form of monomethylvaline for N,N-dimethylvaline in the synthesis of auristatin E (Senter *et al.*, U.S. provisional application no. 60/400,403 filed July 31, 2002, which is incorporated by reference herein in its entirety).

MMAE (100 mg, 0.14 mmol), the linker maleimidocaproyl-L-phenylalanine-L-lysine(MMT)-p-aminobenzyl alcohol p-nitrophenylcarbonate (160 mg, 0.15 mmol, 1.1 eq., prepared as described in Dubowchik *et al.*, *Bioconjugate Chem.* 2002, 13, 855-869), and HOBt (19 mg, 0.14 mmol, 1.0 eq.) were diluted with DMF (2 mL). After 2 min, pyridine (0.5 mL) was added and the reaction mixture was monitored using reverse-phase HPLC. Neither MMAE nor the linker was detected after 24 h. The reaction mixture was concentrated, and the resulting residue was purified using reverse phase preparative-HPLC (Varian Dynamax column 21.4 mm x 25 cm, 5 μ, 100 Å, using a gradient run of MeCN and Et_3N-CO_2 (pH 7) at 20 mL/min from 10% to 100% over 40 min followed by 100% MeCN for 20 min). The relevant fractions were pooled and concentrated to provide an off-white solid intermediate. ES-MS *m/z* 1608.7 $[M+H]^+$

The off-white solid intermediate was diluted with MeCN/water/TFA in an 85:5:10 ratio, respectively. The reaction mixture was monitored using HPLC and was complete in 3 h. The reaction mixture was directly concentrated and the resulting residue was purified using reverse phase preparative-HPLC (Varian Dynamax column 21.4 mm x 25 cm, 5 μ, 100 Å, using a gradient run of MeCN and 0.1% TFA at 20 mL/min from 10%

to 100% over 40 min followed by 100% MeCN for 20 min). The relevant fractions were combined and concentrated to provide the desired drug-linker compound as an off-white powder. Yield: 46 mg (32% overall); ES-MS *m/z* 1334.8 $[M+H]^+$; UV λ_{max} 215, 256 nm.

10.4.2 CONJUGATE PREPARATION

Antibody Reduction. To 4.8 mL cAC10 (10 mg/mL) was added 600 uL of 500 mM sodium borate/500 mM NaCl, pH 8.0, followed by 600 uL of 100 mM DTT in water. After incubation at 37°C for 30 min, the buffer was exchanged by elution over G25 resin equilibrated and eluted with PBS containing 1 mM DTPA (Aldrich). The thiol/Ab value was checked by determining the reduced antibody concentration from the solution's 280 nm absorbance, and the thiol concentration by reaction with DTNB (Aldrich) and determination of the absorbance at 412 nm.

Conjugation of the Reduced Antibody. The reduced mAb was chilled on ice. The drug-linker compound was used as a DMSO solution of known concentration, and the quantity of drug-linker added to the reaction mixture was calculated as follows: L stock solution = V x [Ab] x Fold Excess/[Drug-Linker], where V and [Ab] are the volume and molar concentration of the reduced antibody solution, respectively. 2.3 mL cold PBS/DTPA was added to the reduced antibody solution. 133.6 uL of 7.5 drug-linker compound stock solution was diluted into 1.47 mL acetonitrile. The acetonitrile drug-linker solution was chilled on ice, then added to the reduced antibody solution. The reaction was terminated after 1 hr by the addition of a 20 fold molar excess of cysteine over maleimide. The reaction mixture was concentrated by centrifugal ultrafiltration and purified by elution through de-salting G25 in PBS. cAC10-fkMMAE was then filtered through 0.2 micron filters under sterile conditions and immediately frozen at -80C. cAC10-fkMMAE was analyzed for 1) concentration, by UV absorbance; 2) aggregation, by size exclusion chromatography; 3) drug/Ab, by measuring unreacted thiols with DTNB, and 4) residual free drug, by reverse phase HPLC.

10.5 RESULTS

The ability of cAC10-vcMMAE to inhibit Jurkat T cell proliferation was then examined. Five thousand Jurkat T cells in 100 µl of medium were seeded in 96-well TC plates. Graded concentrations of cAC10-vcMMAE or a control non-binding control IgG (cIgG)-vcMMAE in 100 µl of medium were added to Jurkat cells to achieve the final concentrations given in FIG. 9. After 80 hours of incubation, cellular DNA synthesis was assessed by a 16 hour ^{3}H-TdR pulse. Chimeric AC10-vcMMAE at concentrations higher than 0.001 µg/ml significantly inhibited DNA synthesis. The IC_{50} was approximately 0.1 µg/ml. The control cIgG-vcMMAE did not show significant growth inhibitory action at concentrations lower than 0.1 µg/ml. FIG. 2 shows that soluble, unconjugated cAC10 had growth inhibitory effect on Jurkat cells at concentration higher than 1 µg/ml. Collectively, these data suggest that cAC10 is effective in delivering a cytotoxic drug to $CD30^{+}$ target cells, and that the cAC10-vcMMAE ADC efficiently internalized upon binding to target cells.

11. EXAMPLE 6: ACTIVATION OF NORMAL HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) INDUCED CD30 EXPRESSION ON T LYMPHOCYTES

Human PBMC obtained from normal donors through apheresis were the source of human T lymphocytes. Immobilized anti-CD3 and anti-CD28 mAbs were used to activate T lymphocytes and induce them to proliferate. To immobilize mAbs, one µg/ml of each an anti-CD3 mAb (OKT3) and an anti-CD28 mAb (B-T3, DiaClone Research, Besançon, France, or 9.3, Hara *et al.*, 1985, J. Exp. Med., 161, 1513-1524) in PBS were incubated at 37 C for 2 hours in TC wells or flasks. Unbound mAbs were removed by two washes with PBS. T cell activation was initiated by seeding PBMC into mAb-coated TC wells or flasks at a concentration of $0.5x10^{6}$ cells/ml in culture medium (RPMI-1640, 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids). In some experiments, recombinant human IL-2 (rhIL-2) (Chiron, Emeryville, CA) at a final concentration of 100 IU/ml was also included.

T cell activation and CD30 expression were monitored by multi-color flow cytometric analysis. For detecting CD30 expression, purified anti-CD30 (AC10) was

labeled with the fluorescent probe Alexa Fluor (AF) (Molecular Probes, Eugene, OR) according to the manufacturer's instruction. Before antibody staining, activated PBMC were washed once in staining medium (PBS, 1% bovine serum albumin (BSA), 0.02% sodium azide) and pelleted. A 25 µl cocktail of either AF labeled anti-CD3 + phycoerythrin (PE) labeled anti-CD4 (BD PharMingen, San Diego, CA) + Cy-Chrome (Cy) labeled anti-CD8 (BD PharMingen) or fluorescein isothiocyanate (FITC) anti-CD25 (BD PharMingen) + PE labeled anti-CD4 (BD PharMingen) + Cy labeled anti-CD8 (BD PharMingen) in staining medium was used to resuspend a 0.2 x 10^6 cell pellet. Cells were incubated on ice for 20 minutes, washed 2 times with staining medium, and fixed in 1% paraformaldehyde in PBS before being analyzed on a FACscan (BD Bioscience, San Jose, CA).

As shown in FIG. 10, very few T lymphocytes in unstimulated PBMC expressed CD30. Activation of PBMC with either anti-CD3 + anti-CD28 or anti-CD3 + anti-CD28 + IL-2 induced a time-dependent expression of CD30 on T lymphocytes. In contrast, PBMC maintained in medium alone did not show induction of CD30 expression. Increased expression of CD30 was detected on both $CD4^+$ and $CD4^-$ (containing $CD8^+$ cells, data not shown) cells. The peak of expression on both $CD4^+$ and $CD8^+$ T lymphocytes was around 4 days after stimulation. The level declined thereafter to almost basal after 8 days in culture.

12. EXAMPLE 7: cAC10 ADCS INHIBITED PROLIFERATION OF ACTIVATED NORMAL T LYMPHOCYTES

The ability of cAC10 ADCs to inhibit the proliferation of activated normal T lymphocytes was then examined. Normal PBMCs were activated by anti-CD3 and anti-CD28 mAbs as described in Example 6. On day 3 during culture, which was close to the peak of CD30 induction (FIG. 10), activated PBMC were harvested. A portion of the cells was used for flow cytometric analysis to confirm the CD30 expression on both CD4 and CD8 cells. The rest of the cells were pelleted and resuspended in fresh medium containing 200 IU/ml of rhIL-2 at 50,000 cells/ml. One hundred µl of this cell suspension (5,000 cells) were transferred to each well of 96-well TC plates. One hundred µl of

graded concentrations of cAC10-fkMMAE, cAC10-vcMMAE, or the corresponding non-binding cIgG ADCs in medium supplemented with 200 IU/ml of rhIL-2 were added to culture wells to achieve the concentrations indicated in FIG. 11. Cells were cultured for an additional 48 or 72 hours with a pulse of ^{3}H-TdR during the last 16 hours to assess cellular DNA synthesis. Similar to its activity on Jurkat T cells, cAC10 ADCs inhibited the proliferation of activated PBMC (FIG. 11). Inhibitory effects were detectable when cAC10 ADCs were present at concentrations higher than 0.01 μg/ml. A 72-hour incubation with either ADC resulted in more profound growth inhibition than a 48-hour incubation. Chimeric AC10-fkMMAE appeared to be more active than cAC10-vcMMAE.

13. EXAMPLE 8: MEMORY T LYMPHOCYTES WERE MORE SENSITIVE TO cAC10 ADC THAN NAÏVE T LYMPHOCYTE

As PBMC are made up of different subsets of lymphocytes, the response of one cellular subset toward cAC10 ADCs may easily be masked by other subsets. In order to examine this possibility, total T lymphocytes, naïve and memory T lymphocytes were enriched from PBMC by negative immuno-selection. Briefly, PBMCs were incubated with one of the following antibody cocktails, containing saturating quantities of antibodies, at a final concentration of 20 x 10^{6} cells/ml on ice for 20 minutes. To enrich for total T lymphocytes, the antibody cocktail contained anti-CD14 (BD PharMingen), anti-CD16 (BD PharMingen), and anti-CD20 (BD PharMingen). To enrich for naïve T lymphocytes (CD45RO^{-}), the antibody cocktail contained anti-CD14, anti-CD16, anti-CD20, and anti-CD45RO (BD PharMingen). To enrich for memory T lymphocytes (CD45RA^{-}), the antibody cocktail contained, anti-CD14, anti-CD16, anti-CD20, and anti-CD45RA (BD PharMingen). All antibody cocktails were prepared in medium supplemented with 10% FBS. After antibody binding, cells were washed twice with ice-cold culture medium and resuspended to a concentration of 20x10^{6} cells/ml in culture medium. Antibody-bound cells were removed from the cell suspension by Dynabeads M450 goat anti-mouse lgG paramagnetic beads (Dynal, Oslo, Norway). Before addition to the cell suspension, Dynabeads M450 goat anti-mouse IgG were washed twice by culture medium and resuspended to a concentration of 60x10^{6}/ml in culture medium.

Equal volumes of cell and paramagnetic beads were mixed and rotated for 2 hours at 4°C. The cell/paramagnetic bead suspension was diluted 2-fold with culture medium. Unbound paramagnetic beads and paramagnetic bead-cell conjugates were attracted to the side of the culture by the application of a magnet. Unbound cells, enriched for the subsets described above, were removed. Flow cytometric analysis was conducted to confirm the enrichment as shown on the left column of FIG. 12.

Activation-induced expression of CD30 on different T cell subsets was then examined. Briefly, total, naïve, and memory T lymphocytes obtained from immuno-selection were stimulated by immobilized anti-CD3 and anti-CD28 antibodies in the presence of 200 IU/ml of IL-2 as described in Example 6. After 72 hours of incubation, the expression of CD30 on both CD4 and CD8 cells in the different cultures was determined by flow cytometry, also as outlined in Example 6. Expression of CD30 was detected on both CD4 and CD8 cells regardless of whether the starting population was total, naïve, or memory T lymphocytes (FIG. 12). The levels of CD30 on memory $CD4^+$ and $CD8^+$ cells were slightly higher than the other subsets on a consistent basis.

The responses of activated naïve ($CD45RO^-$) and memory T lymphocytes ($CD45RA^-$) to cAC10 ADCs were then examined. Three-day activated naïve and memory T lymphocytes were plated at 5,000 cells/well in 96-well TC plates in a final volume of 200 µl of medium containing 200 IU/ml of rhIL-2 and cAC10 ADCs or cIgG ADCs at final concentrations indicated in FIG. 13. Cells were incubated for an additional 72 hours with the last 16 hours pulsed with ^{3}H-TdR to assess cellular DNA synthesis. Whereas cIgG ADCs did not have any significant effect on T cell proliferation at concentrations lower than 2 µg/ml, proliferation of activated memory T lymphocytes was significantly inhibited by either cAC10-vcMMAE or cAC10-vcAEFP at concentrations higher than 0.007 µg/ml. By contrast, activated naïve T lymphocytes were relatively refractory to cAC10-vcMMAE. The cAC10-vcAEFP conjugate was found to be more effective than the cAC10-vcMMAE conjugate on both T cell subsets. Notwithstanding, naïve T lymphocytes were still less sensitive to cAC10-vcAEFP than memory T lymphocytes (FIG. 13). The difference in sensitivity between naïve and memory T lymphocytes toward the cAC10 ADCs was probably not a consequence of antigen densities, as both activated naïve and memory T lymphocytes were found to express comparable levels of CD30

(FIG. 12). The growth inhibitory activity of cAC10-vcMMAE reported in FIG. 11 probably reflected the combined responses of naïve and memory T lymphocytes. More importantly, these results suggest that cAC10-vcMMAE may be able to selectively suppress $CD30^+$ memory T lymphocyte proliferation while having minimal effects on $CD30^+$ naïve T lymphocytes. Effector T lymphocytes implicated in the pathogenesis of autoimmune, inflammatory, and allergic diseases are usually antigen-primed, and they belong to the memory T lymphocyte subset. Accordingly, application of cAC10-vcMMAE in therapeutic intervention may have the advantage of only targeting antigen-primed T lymphocytes. On the other hand, application of cAC10-vcAEFP may be preferred in situations in which suppressing the proliferation of both activated naïve and memory T lymphocytes is desired, *e.g.*, during transplant rejection.

14. EXAMPLE 9: ALLOGENEIC STIMULATION OF NORMAL HUMAN T LYMPHOCYTES INDUCED CD30 EXPRESSION

Effector T lymphcytes implicated in the pathogenesis of autoimmune, allergic, and inflammatory responses have usually gone through multiple rounds of antigenic stimulation and expansion. During this process of chronic activation, they continue to carry out effector functions including cytokine secretion and cytolytic responses to induce tissue damages. It is therefore important to examine if T lymphocytes that have undergone repeated rounds of activation and expansion can still express CD30, and how anti-CD30 mAbs and their ADCs can be applied to inhibit the expansion of such chronically activated $CD30^+$ T lymphocytes. One of such T cell lines was generated against allogeneic stimulator cells. $CD4^+$ lymphocytes were enriched from PBMC by the depletion of $CD8^+$ cells as detailed in Example 9. Briefly, PBMC were incubated in culture medium containing a saturating concentration of an anti-CD8 mAb (BD PharMingen) on ice for 20 minutes. Cells were then washed twice with ice-cold culture medium. Anti-CD8 bound cells were removed from the cell suspension by Dynabeads M450 goat anti-mouse IgG paramagnetic beads. Unbound cells, enriched for $CD4^+$ cells, were analyzed by flow cytometry for CD4 expression.

PBMC ($5x10^6$), enriched for $CD4^+$ cells, were co-cultured with an equal number of gamma-irradiated (2700-5000 rads) allogeneic Daudi cells (ATCC). IL-2 was

added to a final concentration of 400 IU/ml on day 4, and the culture was allowed to continue until day 14. Viable cells were then re-stimulated and expanded with irradiated Daudi cells at a T cell to Daudi ratio of 1:3 in the presence of 200 IU/ml of IL-2. This was the beginning of cycle 2 (FIG. 14). After 7 to 9 days in culture, T lymphocytes were re-stimulated again with irradiated Daudi cell to start the following round of expansion. Expression of CD30 was examined 3 to 4 days and 7 to 9 days after the addition of allogeneic stimulator cells. As shown in FIG. 7, CD30 was induced in each round of allogeneic stimulation on both the $CD4^+$ and $CD4^-$ cells. The levels of CD30 expressed gradually decline toward the end of each stimulation cycle. These results confirm that antigen-primed T lymphocytes retain the capacity to express CD30 when they are challenged with antigenic stimulation.

15. EXAMPLE 10: GENERATION OF T LYMPHOCYTE CLONES

An alternative way to simulate effector lymphocytes *in vitro* is to generate T lymphocyte clones. FIG. 16 depicts a protocol for generating antigen non-specific helper (Th) and cytotoxic (Tc) T lymphocytes clones. PBMC were used as the starting material. $CD4^+$ or $CD8^+$ cells were enriched using the immuno-selection methods described in Examples 8 and 9. Two approaches were applied to stimulate and expand T lymphocytes. In the first approach, cells were stimulated with immobilized anti-CD3 plus soluble anti-CD28 and rhIL-2 (200 IU/ml) at limiting dilution in 96-well TC plates. Anti-CD3 was immobilized onto TC wells at one μg/ml as described in Example 6. Alternatively, cells were stimulated with phytohemagglutinin (Sigma, 1-2 μg/ml), 10,000 irradiated CESS cells (ATCC, 2700 rad irradiated), and rhIL-2 (200 IU/ml) at limiting dilution in 96-well plates. Additional supplements, including different combinations of cytokines and/or antibodies against cytokines can be used to skew the development of T lymphocyte clones to Th_1 or Th_2 effector cells.

Clones identified from the limiting dilution assays were further expanded by multiple rounds of re-stimulation; each round lasted for 10 to 12 days. During the second round of expansion, clones were stimulated with PHA (1-2 μg/ml), $1x10^6$ irradiated feeder cells (CESS), and rhIL-2 (200 IU/ml). In some experiments, anti-IL-12 (R&D Systems, 5 μg/ml) and IL-4 (R&D Systems, 10 ng/ml) were supplemented to favor

T lymphocyte clone development toward the Th_2 or Tc_2 subsets. During the subsequent rounds of re-stimulation a T lymphocytes:feeder cells ratio of 1:2 was used and IL-2 was supplemented at 200 IU/ml. Several panels of T cell clones were generated using these stimulation protocols, and they were subjected to further phenotypic and functional analysis.

16. EXAMPLE 11: PHENOTYPE OF T LYMPHOCYTE CLONES

The surface phenotypes of 10 T lymphocyte clones determined by flow cytometry were depicted in FIG. 16. All 10 clones expressed high levels of CD3 and detectable levels of CD28. Six of the 10 clones expressed CD4, and they can be considered as T helper clones (Th). Three of the remaining four can be considered as cytotoxic T lymphocyte clones (Tc) as they expressed CD8. The last clone was $CD4^+/CD8^+$. Expression of CD30 was detectable on all 10 clones. The magnitudes of signals were relatively low, as the analysis was conducted on resting clones.

The T lymphocyte clones were also analyzed for cytokine expression (FIG. 17). Resting clones were stimulated for 4 hours at 37°C with leukocyte activation cocktail (BD PharMingen). Golgi Plug (BD PharMingen) was added to enable accumulation of intracellular cytokine by blocking protein secretion. Cells were then washed and fixed with the Cytofix/Cytoperm Kit (BD PharMingen) according to the manufacturer's instruction. Intracellular cytokines were detected using various anti-cytokine antibodies (BD PharMingen), and stained cells were analyzed by flow cytometric analysis. Two of the Th clones expressed IL-4, IL-13, and IFNγ; this is consistent with a Th_0 profile. Four Th clones showed Th_2 profiles, i.e., they expressed IL-4, IL-5, or IL-13, but not IFNγ. Two Tc clones showed Tc_0 cytokine profile and one Tc clone showed Tc_2 profile. The $CD4^+/CD8^+$ clone expressed IL-4, IL-5, and IL-13, but not IFNγ.

17. EXAMPLE 12: RE-STIMULATION OF T LYMPHOCYTE CLONES INDUCED EXPRESSION OF CD30

The expression of CD30 during re-activation of the T lymphocyte clones was examined. Resting T lymphocyte clones were stimulated with 1 or 2 μg/ml of PHA, irradiated CESS feeder cells at a T:CESS cell ratio of 1:2 to 1:10, 200 IU/ml of IL-2. IL-4

at 20 ng/ml was also supplemented to the Th_2 and Tc_2 clones. The expression of CD25 and CD30 was monitored by flow cytometric analysis. FIG.18 shows the results from one representative Th clone and one representative Tc clone. Extensive upregulation of CD25 was observed in all clones that peaked on day 2. This is indicative of T lymphocyte activation. Expression of CD25 gradually declined in the following days. The induction of CD30 expression paralleled that of CD25. The peak induction was also observed after 2 days of stimulation. Expression was still detectable on day 4 and it gradually declined to almost basal level by day 7. The other 8 clones examined also showed similar kinetics and magnitudes of CD25 and CD30 expression (data not shown). Activation-induced expression of both CD25 and CD30 has been a consistent feature of these T lymphocyte clones.

18. EXAMPLE 13: cAC10 ADCS INHIBITED THE PROLIFERATION OF ACTIVATED T LYMPHOCYTE CLONES

The responses of T lymphocyte clones to cAC10 ADCs were examined next. Resting T lymphocyte clones were activated to express CD30 as described in Example 12. After 2 days of activation, a portion of the cells was analyzed for CD30 expression by flow cytometry to confirm cellular activation and CD30 induction. The remaining cells were pelleted and resuspended in new medium containing 200 IU/ml of rIL-2 or 200 IU/ml of IL-2 and 10 ng/ml IL-4 for the Th_2 and Tc_2 clones. Cells were then plated out at 10,000 cells/well in a final volume of 200 µl of medium containing graded concentrations of cAC10 ADCs or the non-binding IgG ADCs as indicated in FIG. 19 and FIG. 20. Cells were incubated for an additional 48 or 72 hours with the last 16 hours pulsed with ^{3}H-TdR to assess cellular DNA synthesis. Results for the responses of two Th_2 clones toward ADC treatment was shown in FIG. 19 and FIG. 20. Chimeric AC10 ADCs at concentrations higher than 0.01 µg/ml significantly inhibited the proliferation both clones. The control ADC cIgG-fkMMAE did not significantly inhibit proliferation at concentrations below 0.1 µg/ml, whereas for cIgG-vcMMAE concentrations as high as 2 µg/ml showed no growth inhibitory activity, confirming the antigen specificity of the cAC10 ADCs. An incubation of a total of 72 hours also resulted in much profound proliferation inhibition than a 48-hour incubation.

Annexin V binding and membrane permeability to PI described in Example 4 were then used to assess if the inhibition of proliferation effected by the cAC10 ADCs was accompanied by cell death (FIG. 21). After a 48-hour incubation with 1 μg/ml of ADC, 27% of the cAC10-vcMMAE-treated cells was either apoptotic or dead, compared to 7-14% of the cIgG-vcMMAE-treated cells. For the cAC10-fkMMAE-treated cells, 44-48% were apoptotic or dead, compared to 13 and 31% of the cIgG-fkMMAE-treated cells. These data confirmed that the cAC10 ADCs tested were cytotoxic to $CD30^+$ T cell clones.

During the course of analyzing the effects of cAC10 ADCs on the proliferation of activated T lymphocyte clones, several clones were found to be relatively refractory to cAC10-vcMMAE treatment, e.g., clones 3.27.2 and 4.01.1 in FIG. 22. Similar to what was observed in memory and naïve T cells (FIG. 13), an AEFP conjugate of cAC10 showed strong inhibitory activity on the T lymphocyte clones. Thus, both cAC10-fkAEFP and cAC10-vcAEFP conjugates gave IC_{50}'s of 0.01 to 0.1 μg/ml, compared to >1 μg/ml from cAC10-vcMMAE. These data further confirm the utility of different cAC10 ADCs in targeted inhibition of proliferation in T lymphocytes.

19. EXAMPLE 14: ANTIGEN-PRIMED T LYMPHOCYTES WERE MORE SENSITIVE TO cAC10 ADCS

FIG. 23 summarizes the efficacies of cAC10-vcMMAE and cAC10-vcAEFP on inhibiting the proliferation of different types of T lymphocytes. The following trend was observed. First, naïve T cells appeared to be most refractory to both cAC10-vcMMAE and cAC10-vcAEFP when compared to memory T lymphocytes or T lymphocyte clones. Second, between the T lymphocyte clones and memory T cells, eight of the 10 T lymphocyte clones were found to be more sensitive to cAC10-vcMMAE compared to the memory T lymphocytes. These clones have been expanded through multiple rounds of T cell receptor and cytokine stimulation, similar to chronically stimulated effector T cells involved in inflammatory and autoimmune responses. Third, the proliferation of both $CD4^+$ and $CD8^+$ T lymphocytes clones was susceptible to cAC10 ADCs. Fourth, susceptibility to cAC10 ADC also did not appear to correlate to any particular T lymphocyte subsets as defined by their cytokine secretion profiles. Thus, Th_0,

Th_2, and Tc_0 clones and one each of Tc_2 and $CD4^+/CD8^+$ clones were found to be sensitive to cAC10 ADCs. These results suggest that cAC10 ADCs could be used to target multiple T lymphocyte subsets including naïve or memory T lymphocytes, helper ($CD4^+$) or cytotoxic ($CD8^+$) lymphocytes, and effector lymphocytes secreting different combinations of cytokines. Moreover, since effector T lymphocytes participating in the pathogenesis of immune disorders are biologically more similar to the chronically activated T lymphocyte clones than to naïve T cells, cAC10 ADCs may be particularly suited for the targeted depletion of $CD30^+$ effector T lymphocytes involved in the pathogenesis of autoimmune, inflammatory, and allergic responses.

20. SPECIFIC EMBODIMENTS, CITATION OF REFERENCES

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

Various references, including patent applications, patents, and scientific publications, are cited herein, the disclosures of each of which is incorporated herein by reference in its entirety.

WHAT IS CLAIMED IS:

1. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) a first antibody that (i) immunospecifically binds CD30 and (ii) exerts a cytostatic or cytotoxic effect on an activated lymphocyte; and (b) a pharmaceutically acceptable carrier.

2. The method of claim 1, wherein the first antibody is human, humanized or chimeric.

3. The method of claim 1, wherein the first antibody is multivalent.

4. The method of claim 1, wherein the first antibody competes for binding to CD30 with monoclonal antibodies AC10 or HeFi-1.

5. The method of claim 1, wherein the first antibody is capable of exerting the cytotoxic or cytostatic effect without conjugation to a cytotoxic agent.

6. The method of claim 1, wherein the first antibody is capable of exerting the cytotoxic or cytostatic effect in the absence of cells other than the activated lymphocyte.

7. The method of claim 1, wherein the first antibody is capable of exerting the cytotoxic or cytostatic effect as a monospecific antibody.

8. The method of claim 1, further comprising administering an agent that potentiates the cytostatic or cytotoxic effect of the first antibody.

9. The method of claim 1, further comprising administering a second antibody.

10. The method of claim 1, wherein the second antibody recognizes a second receptor or receptor complex expressed on activated lymphocytes.

11. The method of claim 10, wherein the second antibody enhances the cytostatic or cytotoxic effect of the first antibody.

12. The method of claim 11, wherein the second antibody enhances the cytostatic or cytotoxic effect of the first antibody by delivering a signal to the activated lymphocyte.

13. The method of claim 10 or 11, wherein the receptor or the receptor complex comprises an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein.

14. The method of claim 13, wherein the immunoglobulin superfamily member is CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, or ICOS.

15. The method of claim 13, wherein the TNF receptor superfamily member is CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, or APO-3.

16. The method of claim 13, wherein the integrin is CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, or CD104.

17. The method of claim 13, wherein the lectin is C-type, S-type, or I-type lectin.

18. The method of claim 1, wherein the first antibody is a bispecific antibody.

19. The method of claim 18, wherein the wherein the bispecific antibody binds to CD30 and a second receptor or receptor complex expressed on activated lymphocytes.

20. The method of claim 19, wherein the portion of the bispecific antibody that binds to the second receptor or receptor complex enhances the cytostatic or cytotoxic effect of the portion of the bispecific antibody that binds to CD30.

21. The method of claim 20, wherein the binding of the bispecific antibody to the second receptor or receptor complex enhances the cytostatic or cytotoxic effect of the of the portion of the bispecific antibody that binds to CD30 by delivering a signal to the activated lymphocyte.

22. The method of claim 19 or 20, wherein the receptor or receptor complex comprises an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein.

23. The method of claim 22, wherein the immunoglobulin superfamily member is CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, or ICOS.

24. The method of claim 22, wherein the TNF receptor superfamily member is CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, or APO-3.

25. The method of claim 22, wherein the integrin is CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, or CD104.

26. The method of claim 22, wherein the lectin is C-type, S-type, or I-type lectin.

27. The method of claim 1, further comprising administering a ligand that binds to a receptor or receptor complex expressed on activated lymphocytes.

28. The method of claim 27, wherein the ligand enhances the cytostatic or cytotoxic effect of the first antibody.

29. The method of claim 28, wherein the ligand enhances the cytostatic or cytotoxic effect of the first antibody by delivering a signal to the activated lymphocyte.

30. The method of claim 27 or 28, wherein the receptor or receptor complex comprises an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein.

31. The method of claim 30, wherein the immunoglobulin superfamily member is CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, or ICOS.

32. The method of claim 30, wherein the TNF receptor superfamily member is CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, or APO-3.

33. The method of claim 30, wherein the integrin is CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, or CD104.

34. The method of claim 30, wherein the lectin is C-type, S-type, or I-type lectin.

35. The method of claim 1, wherein the first antibody is a fusion protein comprising the amino acid sequence of a second protein that is not an antibody.

36. The method of claim 35, wherein the second protein confers multivalent binding properties to the first antibody.

37. The method of claim 1, 9, 10, 18, or 27, further comprising administering an immunosuppressive agent.

38. The method of claim 37, wherein the immunosuppressive agent is gancyclovir, etanercept, cyclosporine, tacrolimus, or rapamycin.

39. The method of claim 37, wherein the immunosuppressive agent is an alkylating agent.

40. The method of claim 39, wherein the alkylating agent is cyclophosphamide.

41. The method of claim 37, wherein the immunosuppressive agent is an antimetabolite.

42. The method of claim 41, wherein the antimetabolite is a purine antagonist.

43. The method of claim 42, wherein the purine antagonist is azathioprine, or mycophenolate mofetil.

44. The method of claim 41, wherein the antimetabolite is a dihydrofolate reductase inhibitor.

45. The method of claim 44, wherein the dihydrofolate reductase inhibitor is methotrexate.

46. The method of claim 37, wherein the immunosuppressive agent is a glucocorticoid.

47. The method of claim 46, wherein the glucocorticoid is cortisol or aldosterone.

48. The method of claim 37, wherein the immunosuppressive agent is a glucocorticoid analogue.

49. The method of claim 48, wherein the glucocorticoid analogue is prednisone or dexamethasone.

50. The method of claim 37, wherein the immunosuppressive agent is an anti-inflammatory agent.

51. The method of claim 50, wherein the anti-inflammatory agent is a cyclooxygenase inhibitor, a 5-lipoxygenase inhibitor, or a leukotriene receptor antagonist.

52. The method of claim 1, wherein the first antibody is conjugated to a cytotoxic agent.

53. The method of claim 52, wherein the cytotoxic agent is selected from the group consisting of an enediyne, a lexitropsin, a duocarmycin, a taxane, a puromycin, a dolastatin, a maytansinoid, a DNA minor groove binding agent, a DNA minor groove alkylating agent, and a vinca alkaloid.

54. The method of claim 52, wherein the cytotoxic agent is paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretastatin, calicheamicin, maytansine, DM-1, auristatin E, AEB, AEVB, AEFP, MMAE, or netropsin.

55. The method of claim 52, wherein the cytotoxic agent is an anti-tubulin agent.

56. The method of claim 55, wherein the cytotoxic agent is a vinca alkaloid, a podophyllotoxin, a taxane, a baccatin derivative, a cryptophysin, a maytansinoid, a combretastatin, or a dolastatin.

57. The method of claim 55, wherein the cytotoxic agent is vincristine, vinblastine, vindesine, vinorelbine, VP-16, camptothecin, paclitaxel, docetaxel, epithilone A, epithilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, maytansine, DM-1, auristatin E, AEB, AEVB, AEFP, MMAE, or eleutherobin.

58. The method of claim 52, wherein the cytotoxic agent is MMAE.

59. The method of claim 52, wherein the cytotoxic agent is AEFP.

60. The method of claim 52, wherein the first antibody is conjugated to the cytotoxic agent via a peptide linker.

61. The method of claim 52, wherein the first antibody is conjugated to the cytotoxic agent via a val-cit linker or a phe-lys linker.

62. The method of claim 52, wherein the first antibody is conjugated to the cytotoxic agent via a hydrazone-linker, or a disulfide-linker.

63. The method of claim 52, wherein the conjugate is cAC10-val-cit-MMAE.

64. The method of claim 52, wherein the conjugate is cAC10-val-cit-AEFP.

65. The method of claim 52, wherein the first antibody is conjugated to the cytotoxic agent via a linker that is hydrolyzable at a pH of less than 5.5.

66. The method of claim 65, wherein the linker is hydrolyzable at a pH of less than 5.0.

67. The method of claim 65, wherein the linker is a hydrazone linker or a disulfide linker.

68. The method of claim 52, wherein the first antibody is conjugated to the cytotoxic agent via a linker, wherein the linker is cleavable by a protease.

69. The method of claim 52, wherein the first antibody is conjugated to the cytotoxic agent via a peptide linker, and wherein the linker is cleavable by a protease.

70. The method of claim 68, wherein the protease is a membrane-associated protease.

71. The method of claim 68, wherein the protease is an intracellular protease.

72. The method of claim 68, wherein the protease is an endosomal protease.

73. The method of claim 68, wherein the protease is a lysosomal protease.

74. The method of claim 52, wherein the first antibody is a monoclonal antibody, a chimeric antibody, a human antibody, a humanized antibody, a glycosylated antibody, a multispecific antibody, a single-chain antibody, a Fab fragment, a F(ab') fragment, a $F(ab')_2$ fragment, a Fd, a single-chain Fv, a disulfide-linked Fv, a fragment comprising a V_L domain, a polypeptide that binds specifically to CD30, or a fragment comprising a V_H domain.

75. The method of claim 1, wherein the first antibody is conjugated to a immunosuppressive agent.

76. The method of claim 75, wherein the immunosuppressive agent is gancyclovir, etanercept, cyclosporine, tacrolimus, or rapamycin.

77. The method of claim 75, wherein the immunosuppressive agent is an alkylating agent.

78. The method of claim 77, wherein the alkylating agent is cyclophosphamide.

79. The method of claim 75, wherein the immunosuppressive agent is an antimetabolite.

80. The method of claim 79, wherein the antimetabolite is a purine antagonist.

81. The method of claim 80, wherein the purine antagonist is azathioprine, or mycophenolate mofetil.

82. The method of claim 79, wherein the antimetabolite is a dihydrofolate reductase inhibitor.

83. The method of claim 82, wherein the dihydrofolate reductase inhibitor is methotrexate.

84. The method of claim 75, wherein the immunosuppressive agent is a glucocorticoid.

85. The method of claim 84, wherein the glucocorticoid is cortisol or aldosterone.

86. The method of claim 75, wherein the immunosuppressive agent is a glucocorticoid analogue.

87. The method of claim 86, wherein the glucocorticoid analogue is prednisone or dexamethasone.

88. The method of claim 75, wherein the immunosuppressive agent is an anti-inflammatory agent.

89. The method of claim 88, wherein the anti-inflammatory agent is a cyclooxygenase inhibitor, a 5-lipoxygenase inhibitor, or a leukotriene receptor antagonist.

90. The method of claim 1, wherein the immunological disorder is a Th_2-lymphocyte related disorder.

91. The method of claim 90, wherein the immunological disorder is atopic dermatitis, systemic lupus erythematosus, atopic asthma, rhinoconjunctivitis, allergic rhinitis, Omenn's syndrome, systemic sclerosis, or chronic graft versus host disease.

92. The method of claim 1, wherein the immunological disorder is a Th_1 lymphocyte-related disorder.

93. The method of claim 92, wherein the immunological disorder is rheumatoid arthritis, multiple sclerosis, psoriasis, Sjorgren's syndrome, Hashimoto's thyroiditis, Grave's disease, primary biliary cirrhosis, Wegener's granulomatosis, tuberculosis, or acute graft versus host disease.

94. The method of claim 1, wherein the immunological disorder is due to viral infection.

95. The method of claim 94, wherein the viral infection involves the Epstein-Barr virus, human immunodeficiency virus, human T leukemia virus, hepatitis B virus, or measles virus.

96. The method of claim 1, wherein the immunological disorder is an activated B lymphocyte-related disorder.

97. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) a first antibody that (i) immunospecifically binds CD30 and (ii) induces CD30 signaling in a lymphocyte; and (b) a pharmaceutically acceptable carrier.

98. The method of claim 97, wherein the first antibody is human, humanized or chimeric.

99. The method of claim 97, wherein the first antibody is multivalent.

100. The method of claim 97, wherein the first antibody competes for binding to CD30 with monoclonal antibodies AC10 or HeFi-1.

101. The method of claim 97, wherein the first antibody is capable of inducing CD30 signaling in the absence of cells other than the lymphocyte.

102. The method of claim 97, wherein the first antibody is capable of inducing CD30 signaling as a monospecific antibody.

103. The method of claim 97, further comprising administering a second antibody.

104. The method of claim 97, wherein the second antibody recognizes a second receptor or receptor complex expressed on activated lymphocytes.

105. The method of claim 104, wherein the receptor or the receptor complex comprises an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein.

106. The method of claim 105, wherein the immunoglobulin superfamily member is CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, or ICOS.

107. The method of claim 105, wherein the TNF receptor superfamily member is CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, or APO-3.

108. The method of claim 105, wherein the integrin is CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, or CD104.

109. The method of claim 105, wherein the lectin is C-type, S-type, or I-type lectin.

110. The method of claim 97, wherein the first antibody is a bispecific antibody.

111. The method of claim 110, wherein the wherein the bispecific antibody binds to CD30 and a second receptor or receptor complex expressed on activated lymphocytes.

112. The method of claim 111, wherein the receptor or receptor complex comprises an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein.

113. The method of claim 112, wherein the immunoglobulin superfamily member is CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, or ICOS.

114. The method of claim 112, wherein the TNF receptor superfamily member is CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, or APO-3.

115. The method of claim 112, wherein the integrin is CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, or CD104.

116. The method of claim 112, wherein the lectin is C-type, S-type, or I-type lectin.

117. The method of claim 97, further comprising administering a ligand that binds to a receptor or receptor complex expressed on activated lymphocytes.

118. The method of claim 117, wherein the receptor or receptor complex comprises an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein.

119. The method of claim 118, wherein the immunoglobulin superfamily member is CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA-4, PD-1, or ICOS.

120. The method of claim 118, wherein the TNF receptor superfamily member is CD27, CD40, CD95/Fas, CD134/OX40, CD137/4-1BB, TNF-R1, TNFR-2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL-R1, TRAIL-R2, TRAIL-R3, TRAIL-R4, or APO-3.

121. The method of claim 118, wherein the integrin is CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, or CD104.

122. The method of claim 118, wherein the lectin is C-type, S-type, or I-type lectin.

123. The method of claim 97, wherein the first antibody is a fusion protein comprising the amino acid sequence of a second protein that is not an antibody.

124. The method of claim 123, wherein the second protein confers multivalent binding properties to the first antibody.

125. The method of claim 97, 103, 104, 110, or 111, further comprising administering an immunosuppressive agent.

126. The method of claim 125, wherein the immunosuppressive agent is gancyclovir, etanercept, cyclosporine, tacrolimus, or rapamycin.

127. The method of claim 125, wherein the immunosuppressive agent is an alkylating agent.

128. The method of claim 127, wherein the alkylating agent is cyclophosphamide.

129. The method of claim 125, wherein the immunosuppressive agent is an antimetabolite.

130. The method of claim 129, wherein the antimetabolite is a purine antagonist.

131. The method of claim 130, wherein the purine antagonist is azathioprine, or mycophenolate mofetil.

132. The method of claim 129, wherein the antimetabolite is a dihydrofolate reductase inhibitor.

133. The method of claim 132, wherein the dihydrofolate reductase inhibitor is methotrexate.

134. The method of claim 125, wherein the immunosuppressive agent is a glucocorticoid.

135. The method of claim 134, wherein the glucocorticoid is cortisol or aldosterone.

136. The method of claim 125, wherein the immunosuppressive agent is a glucocorticoid analogue.

137. The method of claim 136, wherein the glucocorticoid analogue is prednisone or dexamethasone.

138. The method of claim 125, wherein the immunosuppressive agent is an anti-inflammatory agent.

139. The method of claim 138, wherein the anti-inflammatory agent is a cyclooxygenase inhibitor, a 5-lipoxygenase inhibitor, or a leukotriene receptor antagonist.

140. The method of claim 97, wherein the first antibody is conjugated to a cytotoxic agent.

141. The method of claim 140, wherein the cytotoxic agent is selected from the group consisting of an enediyne, a lexitropsin, a duocarmycin, a taxane, a puromycin, a dolastatin, a maytansinoid, a DNA minor groove binding agent, a DNA minor groove alkylating agent, and a vinca alkaloid.

142. The method of claim 140, wherein the cytotoxic agent is paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretastatin, calicheamicin, maytansine, DM-1, auristatin E, AEB, AEVB, AEFP, MMAE, or netropsin.

143. The method of claim 140, wherein the cytotoxic agent is an anti-tubulin agent.

144. The method of claim 143, wherein the cytotoxic agent is a vinca alkaloid, a podophyllotoxin, a taxane, a baccatin derivative, a cryptophysin, a maytansinoid, a combretastatin, or a dolastatin.

145. The method of claim 143, wherein the cytotoxic agent is vincristine, vinblastine, vindesine, vinorelbine, VP-16, camptothecin, paclitaxel, docetaxel, epithilone A, epithilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, maytansine, DM-1, auristatin E, AEB, AEVB, AEFP, MMAE, or eleutherobin.

146. The method of claim 140, wherein the cytotoxic agent is MMAE.

147. The method of claim 140, wherein the cytotoxic agent is AEFP.

148. The method of claim 140, wherein the first antibody is conjugated to the cytotoxic agent via a peptide linker.

149. The method of claim 140, wherein the first antibody is conjugated to the cytotoxic agent via a val-cit linker or a phe-lys linker.

150. The method of claim 140, wherein the first antibody is conjugated to the cytotoxic agent via a hydrazone-linker, or a disulfide-linker.

151. The method of claim 140, wherein the first antibody is conjugated to the cytotoxic agent via a linker that is hydrolyzable at a pH of less than 5.5.

152. The method of claim 151, wherein the linker is hydrolyzable at a pH of less than 5.0.

153. The method of claim 151, wherein the linker is a hydrazone linker or a disulfide linker.

154. The method of claim 140, wherein the first antibody is conjugated to the cytotoxic agent via a linker, wherein the linker is cleavable by a protease.

155. The method of claim 140, wherein the first antibody is conjugated to the cytotoxic agent via a peptide linker, and wherein the linker is cleavable by a protease.

156. The method of claim 154, wherein the protease is a membrane-associated protease.

157. The method of claim 154, wherein the protease is an intracellular protease.

158. The method of claim 154, wherein the protease is an endosomal protease.

159. The method of claim 154, wherein the protease is a lysosomal protease.

160. The method of claim 140, wherein the first antibody is a monoclonal antibody, a chimeric antibody, a human antibody, a humanized antibody, a glycosylated antibody, a multispecific antibody, a single-chain antibody, a Fab fragment, a F(ab') fragment, a $F(ab')_2$ fragment, a Fd, a single-chain Fv, a disulfide-linked Fv, a fragment comprising a V_L domain, a polypeptide that binds specifically to CD30, or a fragment comprising a V_H domain.

161. The method of claim 97, wherein the first antibody is conjugated to a immunosuppressive agent.

162. The method of claim 161, wherein the immunosuppressive agent is gancyclovir, etanercept, cyclosporine, tacrolimus, or rapamycin.

163. The method of claim 161, wherein the immunosuppressive agent is an alkylating agent.

164. The method of claim 163, wherein the alkylating agent is cyclophosphamide.

165. The method of claim 161, wherein the immunosuppressive agent is an antimetabolite.

166. The method of claim 165, wherein the antimetabolite is a purine antagonist.

167. The method of claim 166, wherein the purine antagonist is azathioprine, or mycophenolate mofetil.

168. The method of claim 165, wherein the antimetabolite is a dihydrofolate reductase inhibitor.

169. The method of claim 168, wherein the dihydrofolate reductase inhibitor is methotrexate.

170. The method of claim 161, wherein the immunosuppressive agent is a glucocorticoid.

171. The method of claim 170, wherein the glucocorticoid is cortisol or aldosterone.

172. The method of claim 161, wherein the immunosuppressive agent is a glucocorticoid analogue.

173. The method of claim 172, wherein the glucocorticoid analogue is prednisone or dexamethasone.

174. The method of claim 161, wherein the immunosuppressive agent is an anti-inflammatory agent.

175. The method of claim 174, wherein the anti-inflammatory agent is a cyclooxygenase inhibitor, a 5-lipoxygenase inhibitor, or a leukotriene receptor antagonist.

176. The method of claim 97, wherein the immunological disorder is a Th_2-lymphocyte related disorder.

177. The method of claim 176, wherein the immunological disorder is atopic dermatitis, systemic lupus erythematosus, atopic asthma, rhinoconjunctivitis, allergic rhinitis, Omenn's syndrome, systemic sclerosis, or chronic graft versus host disease.

178. The method of claim 97, wherein the immunological disorder is a Th_1 lymphocyte-related disorder.

179. The method of claim 178, wherein the immunological disorder is rheumatoid arthritis, multiple sclerosis, psoriasis, Sjorgren's syndrome, Hashimoto's thyroiditis, Grave's disease, primary biliary cirrhosis, Wegener's granulomatosis, tuberculosis, or acute graft versus host disease.

180. The method of claim 97, wherein the immunological disorder is due to viral infection.

181. The method of claim 180, wherein the viral infection involves the Epstein-Barr virus, human immunodeficiency virus, human T leukemia virus, hepatitis B virus, or measles virus.

182. The method of claim 97, wherein the immunological disorder is an activated B lymphocyte-related disorder.

183. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1; and (b) a pharmaceutically acceptable carrier.

184. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises SEQ ID NO:2; and (b) a pharmaceutically acceptable carrier.

185. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises one, two or all of: SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:8; and (b) a pharmaceutically acceptable carrier.

186. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises SEQ ID NO:18; and (b) a pharmaceutically acceptable carrier.

187. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the

subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) comprises one, two or all of: SEQ ID NO:20, SEQ ID NO:22 and SEQ ID NO:24; and (b) a pharmaceutically acceptable carrier.

188. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) an antibody that (i) immunospecifically binds CD30 and (ii) competes for binding to CD30 with monoclonal antibody AC10 or HeFi-1, wherein said antibody is conjugated to a cytotoxic agent; and (b) a pharmaceutically acceptable carrier.

189. The method of claim 188, wherein the cytotoxic agent is selected from the group consisting of an enediyne, a lexitropsin, a duocarmycin, a taxane, a puromycin, a dolastatin, a maytansinoid, a DNA minor groove binding agent, a DNA minor groove alkylating agent, and a vinca alkaloid.

190. The method of claim 188, wherein the cytotoxic agent is paclitaxel, docetaxel, CC-1065, SN-38, topotecan, morpholino-doxorubicin, rhizoxin, cyanomorpholino-doxorubicin, dolastatin-10, echinomycin, combretastatin, calicheamicin, maytansine, DM-1, auristatin E, AEB, AEVB, AEFP, MMAE, or netropsin.

191. The method of claim 188, wherein the cytotoxic agent is an anti-tubulin agent.

192. The method of claim 191, wherein the cytotoxic agent is a vinca alkaloid, a podophyllotoxin, a taxane, a baccatin derivative, a cryptophysin, a maytansinoid, a combretastatin, or a dolastatin.

193. The method of claim 191, wherein the cytotoxic agent is vincristine, vinblastine, vindesine, vinorelbine, VP-16, camptothecin, paclitaxel, docetaxel, epithilone A, epithilone B, nocodazole, colchicine, colcimid, estramustine, cemadotin, discodermolide, maytansine, DM-1, auristatin E, AEB, AEVB, AEFP, MMAE, or eleutherobin.

194. The method of claim 188, wherein the cytotoxic agent is MMAE.

195. The method of claim 188, wherein the cytotoxic agent is AEFP.

196. The method of claim 188, wherein the first antibody is conjugated to the cytotoxic agent via a peptide linker.

197. The method of claim 188, wherein the first antibody is conjugated to the cytotoxic agent via a val-cit linker or a phe-lys linker.

198. The method of claim 188, wherein the first antibody is conjugated to the cytotoxic agent via a hydrazone-linker, or a disulfide-linker.

199. The method of claim 188, wherein the first antibody is conjugated to the cytotoxic agent via a linker that is hydrolyzable at a pH of less than 5.5.

200. The method of claim 199, wherein the linker is hydrolyzable at a pH of less than 5.0.

201. The method of claim 199, wherein the linker is a hydrazone linker or a disulfide linker.

202. The method of claim 188, wherein the first antibody is conjugated to the cytotoxic agent via a linker, wherein the linker is cleavable by a protease.

203. The method of claim 188, wherein the first antibody is conjugated to the cytotoxic agent via a peptide linker, and wherein the linker is cleavable by a protease.

204. The method of claim 202, wherein the protease is a membrane-associated protease.

205. The method of claim 202, wherein the protease is an intracellular protease.

206. The method of claim 202, wherein the protease is an endosomal protease.

207. The method of claim 202, wherein the protease is a lysosomal protease.

208. The method of claim 188, wherein the first antibody is a monoclonal antibody, a chimeric antibody, a human antibody, a humanized antibody, a glycosylated antibody, a multispecific antibody, a single-chain antibody, a Fab fragment, a F(ab') fragment, a F(ab')2 fragment, a Fd, a single-chain Fv, a disulfide-linked Fv, a fragment comprising a VL domain, a polypeptide that binds specifically to CD30, or a fragment comprising a VH domain.

209. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) cAC10-val-cit-MMAE; and (b) a pharmaceutically acceptable carrier.

210. A method for the treatment of an immunological disorder in a subject, wherein the immunological disorder is not cancer, comprising administering to the subject, in an amount effective for said treatment, a pharmaceutical composition comprising (a) cAC10-val-cit-AEFP; and (b) a pharmaceutically acceptable carrier.

FIG. 1

	Mean fluorescence intensity
IgG	6
CD3	152
CD4	16
CD28	21
CD30	30

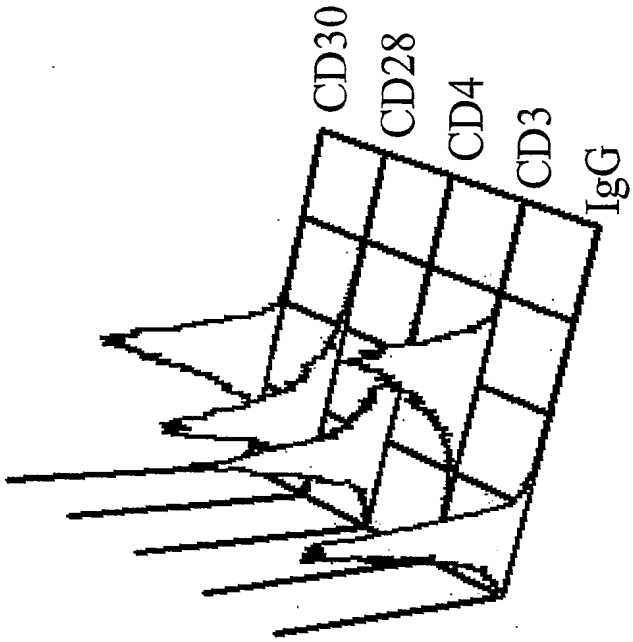


FIG. 2

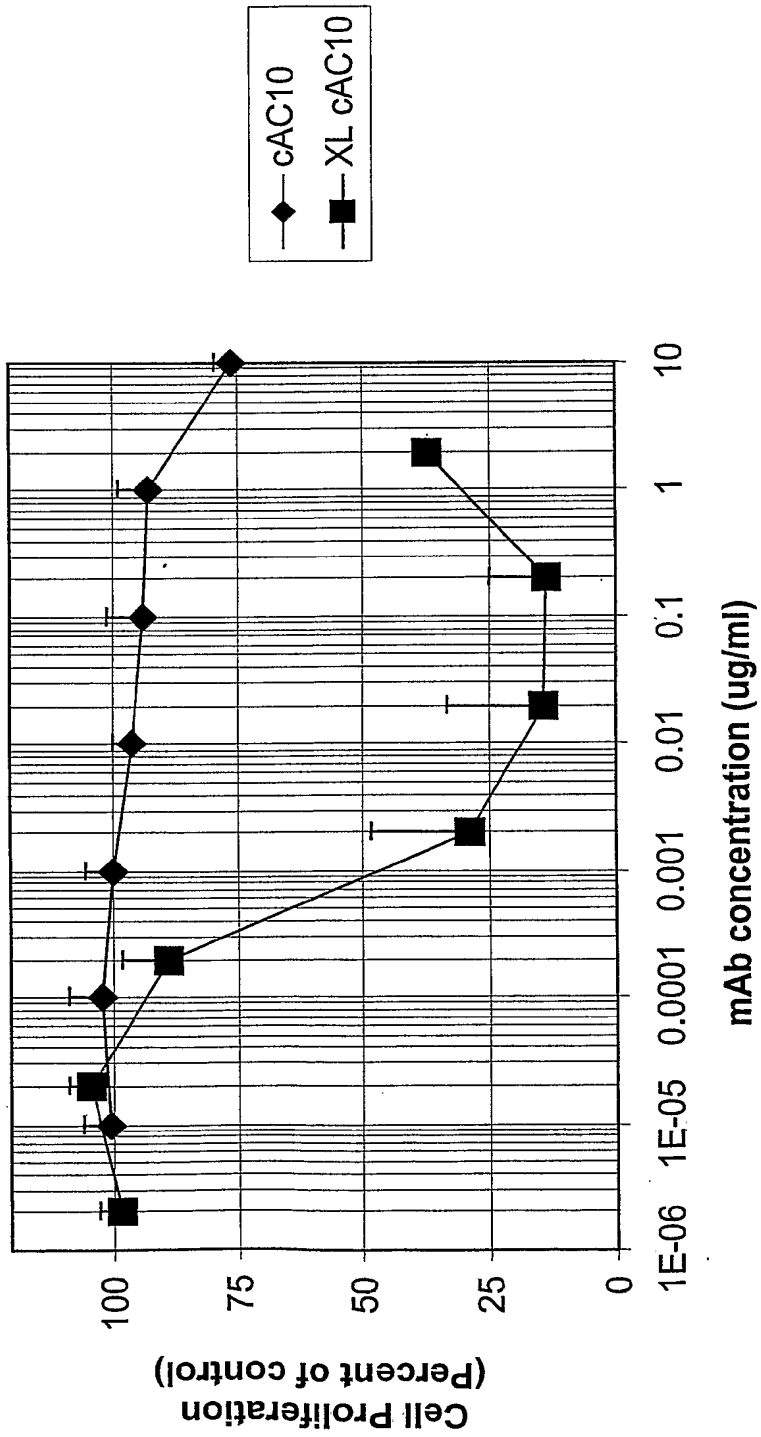

cAC10
XL cAC10
Cell Proliferation
(Percent of control)
100
75
50
25
0
1E-06
1E-05
0.0001
0.001
0.01
0.1
1
10
mAb concentration (ug/ml)

FIG. 3

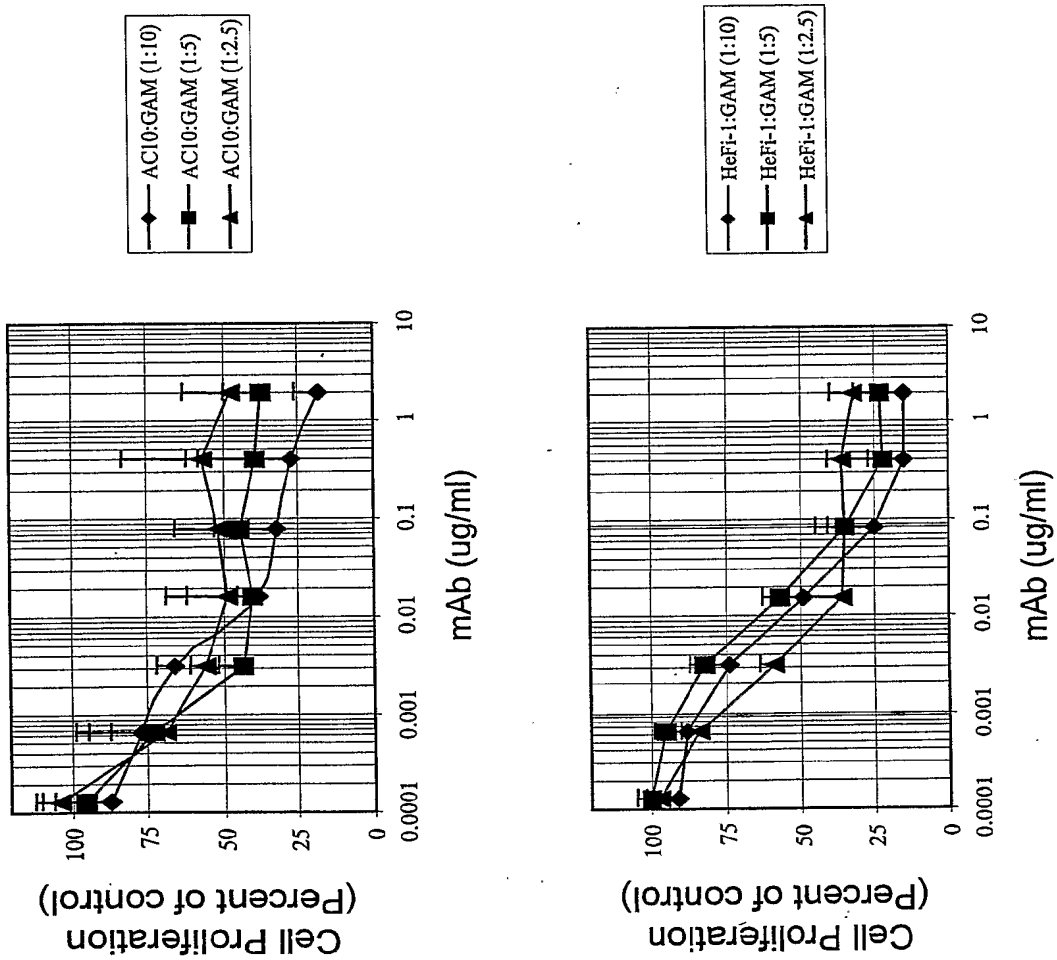

AC10:GAM (1:10)
AC10:GAM (1:5)
AC10:GAM (1:2.5)
Cell Proliferation
(Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
mAb (ug/ml)
HeFi-1:GAM (1:10)
HeFi-1:GAM (1:5)
HeFi-1:GAM (1:2.5)
Cell Proliferation
(Percent of control)
mAb (ug/ml)

FIG. 4

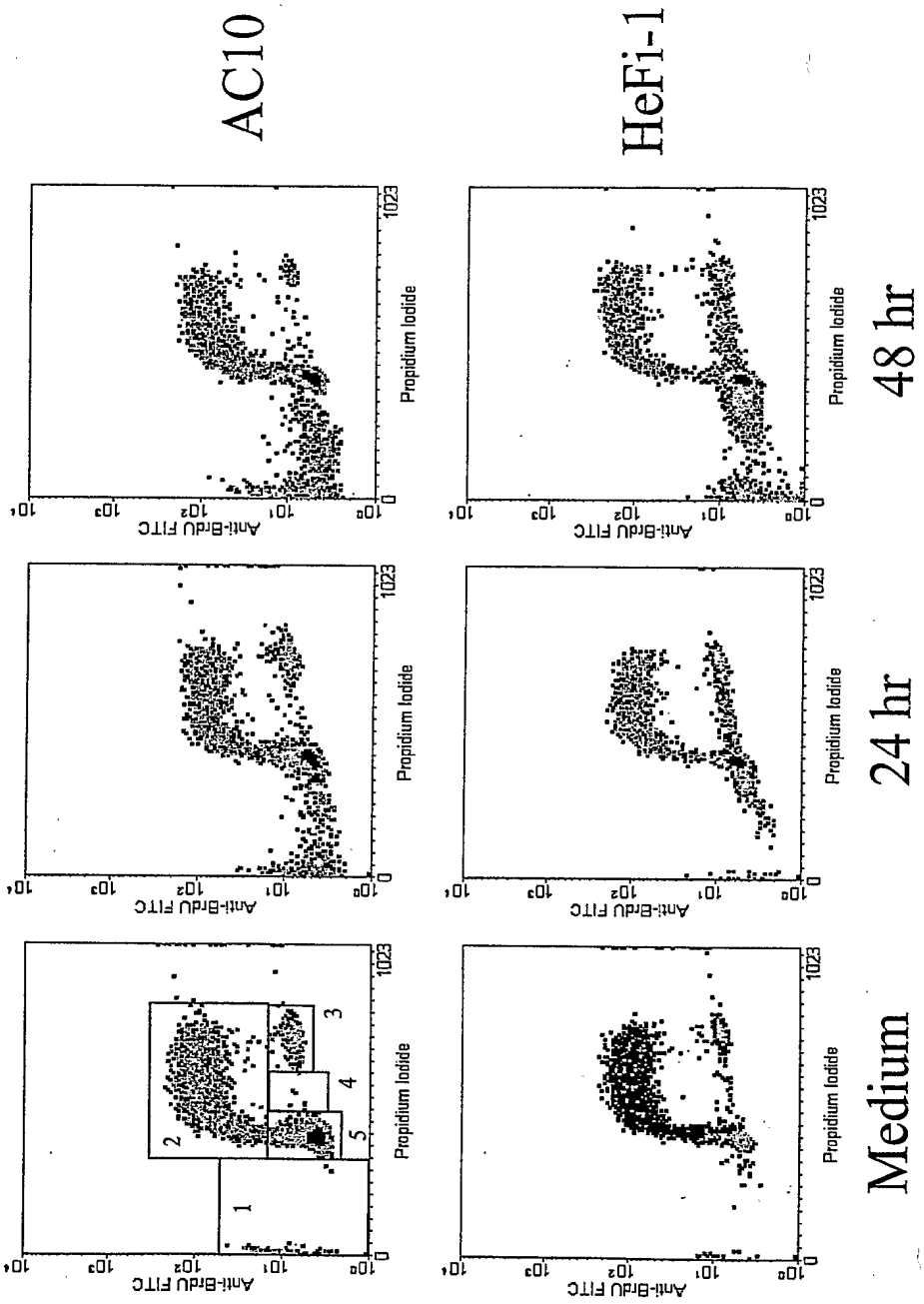

AC10
HeFi-1
Medium
24 hr
48 hr
Anti-BrdU FITC
Propidium Iodide
1. Apoptotic
2. S phase
3. G_2/M phase
4. Apoptotic
5. G_0/G_1 phase

FIG. 5

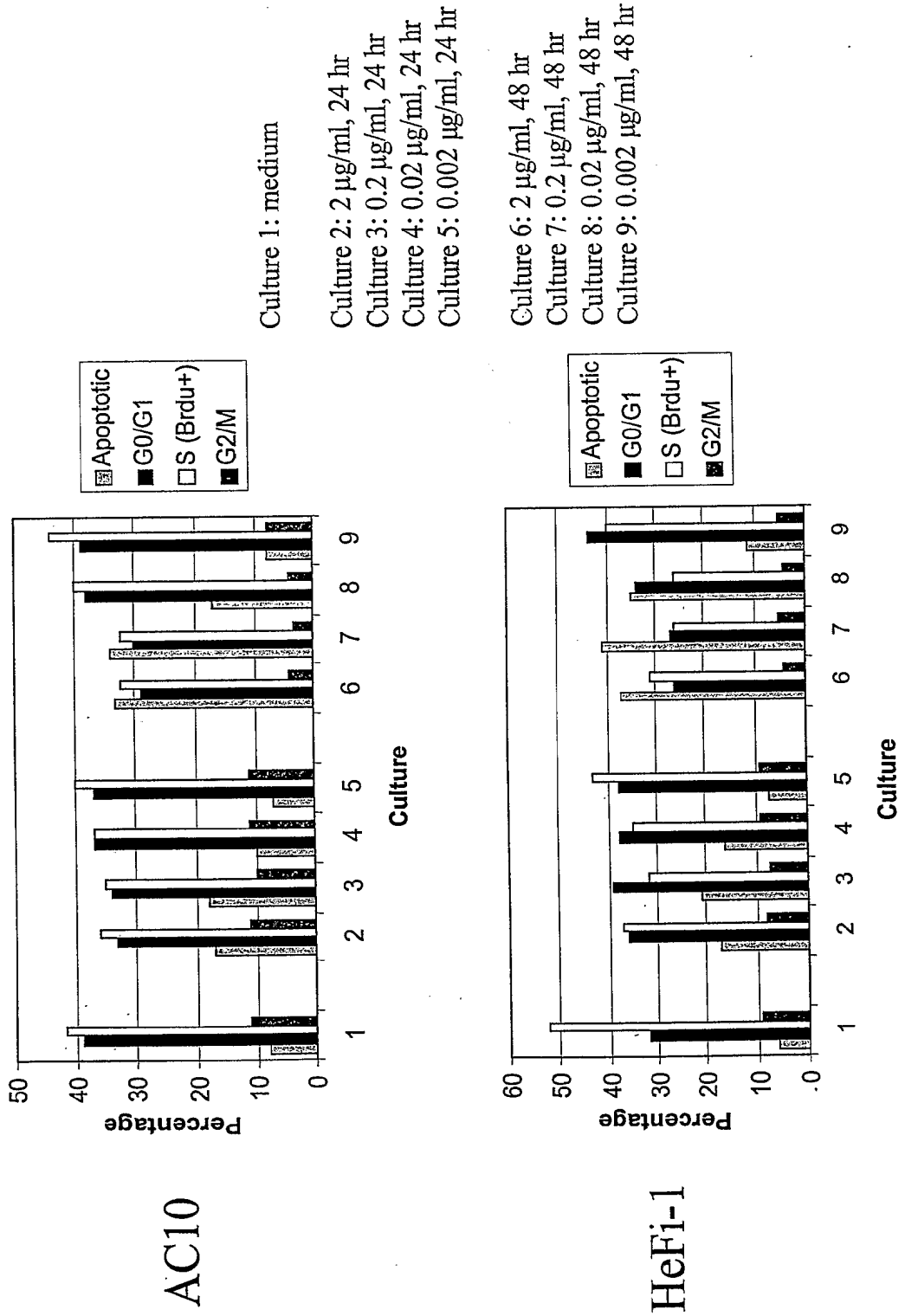

Culture 1: medium
Culture 2: 2 µg/ml, 24 hr
Culture 3: 0.2 µg/ml, 24 hr
Culture 4: 0.02 µg/ml, 24 hr
Culture 5: 0.002 µg/ml, 24 hr
Culture 6: 2 µg/ml, 48 hr
Culture 7: 0.2 µg/ml, 48 hr
Culture 8: 0.02 µg/ml, 48 hr
Culture 9: 0.002 µg/ml, 48 hr
Apoptotic
G0/G1
S (Brdu+)
G2/M
Percentage
Culture
AC10
HeFi-1

FIG. 6

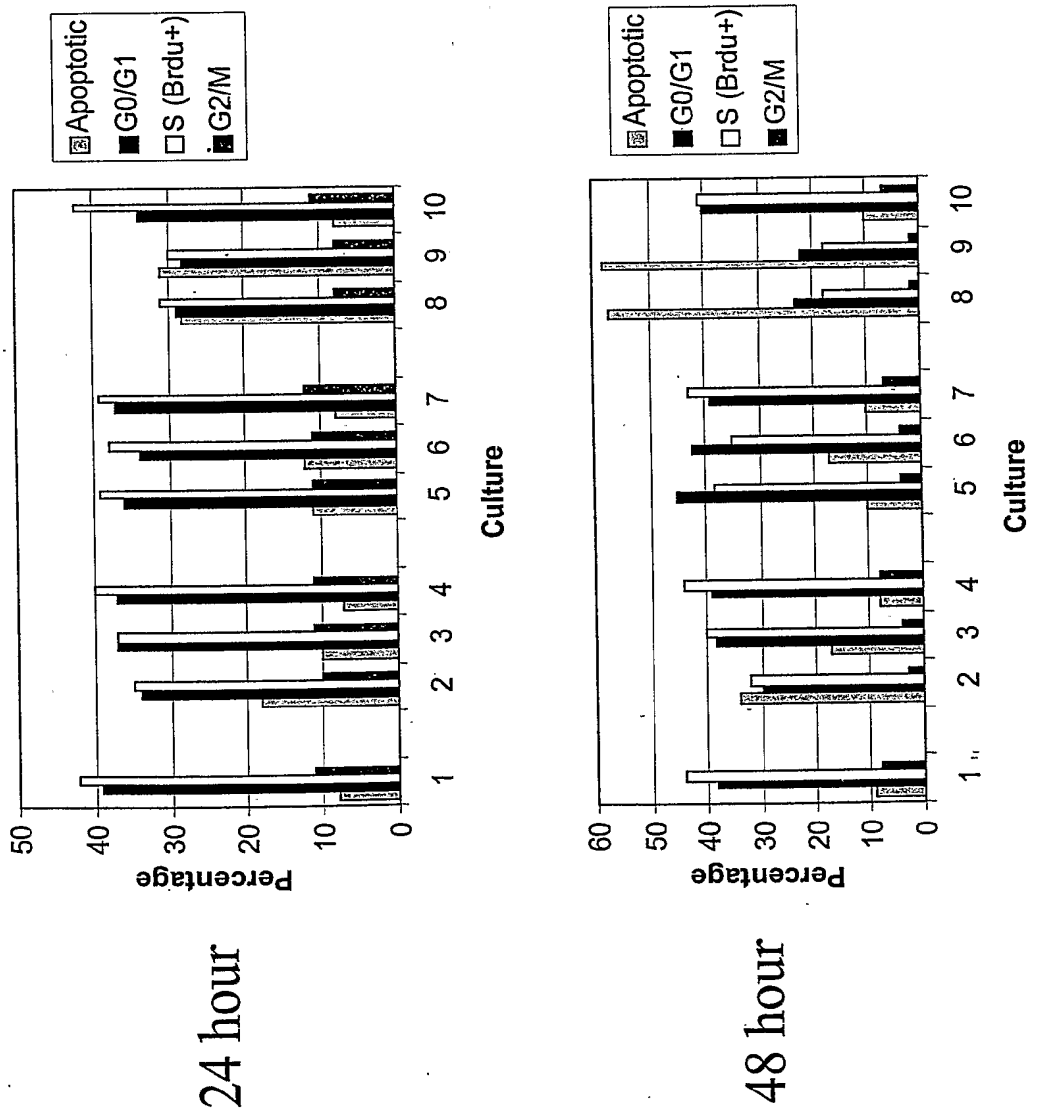

24 hour
Apoptotic
G0/G1
S (Brdu+)
G2/M
Percentage
Culture
Culture 1: medium
Culture 2: 2 µg/ml AC10
Culture 3: 0.2 µg/ml AC10
Culture 4: 0.02 µg/ml AC10
Culture 5: 2 µg/ml OKT3
Culture 6: 0.2 µg/ml OKT3
Culture 7: 0.02 µg/ml OKT3
48 hour
Apoptotic
G0/G1
S (Brdu+)
G2/M
Percentage
Culture
Culture 8: 2 µg/ml AC10 + 2 µg/ml OKT3
Culture 9: 0.2 µg/ml AC10 + 0.2 µg/mll OKT3
Culture 10: 0.02 µg/ml AC10 + 0.02 µg/ml OKT3

FIG. 7

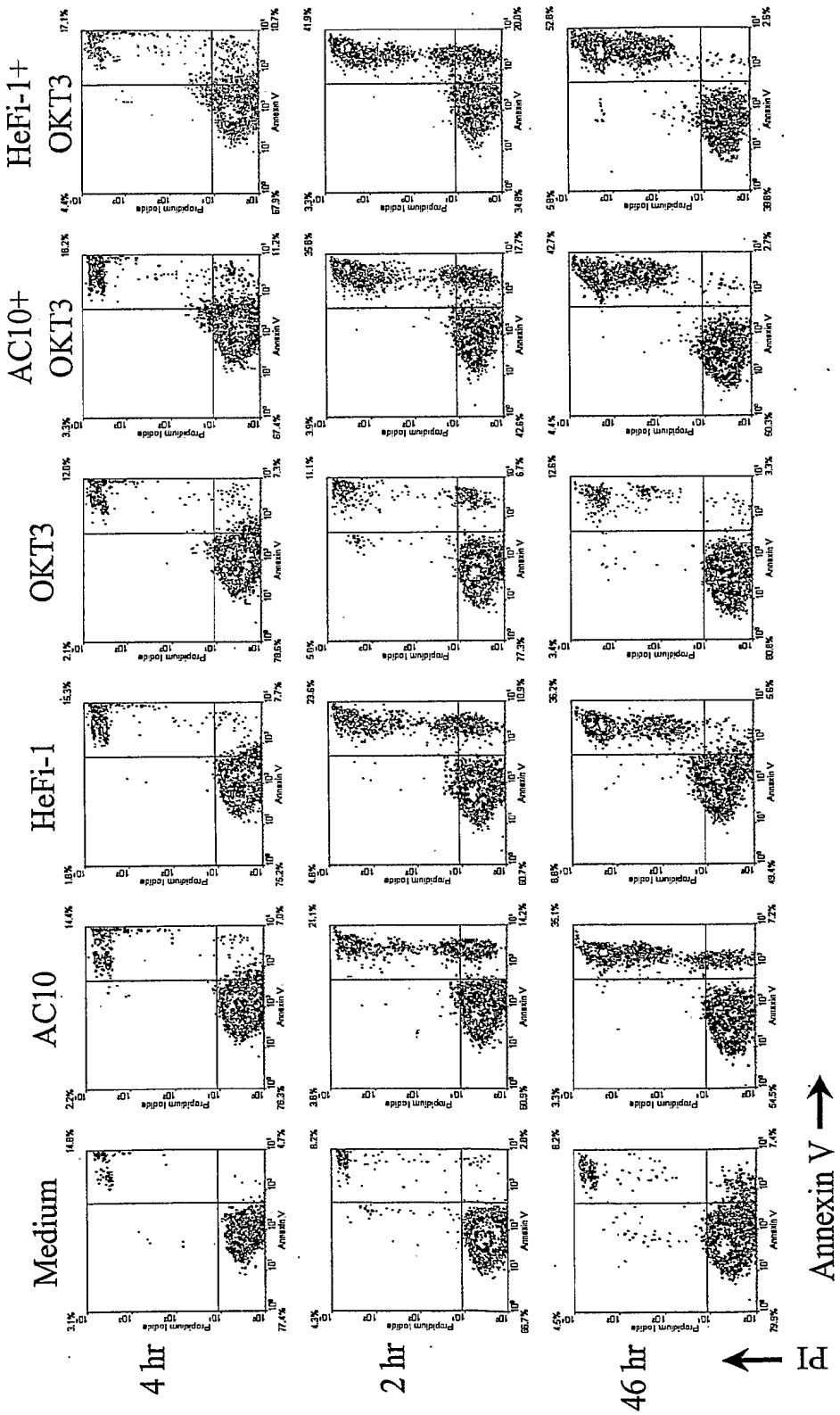
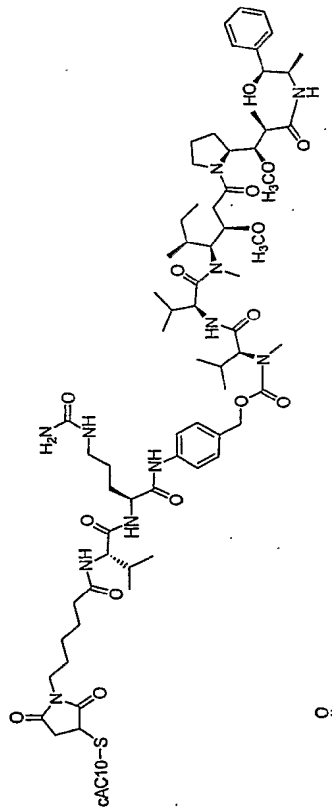
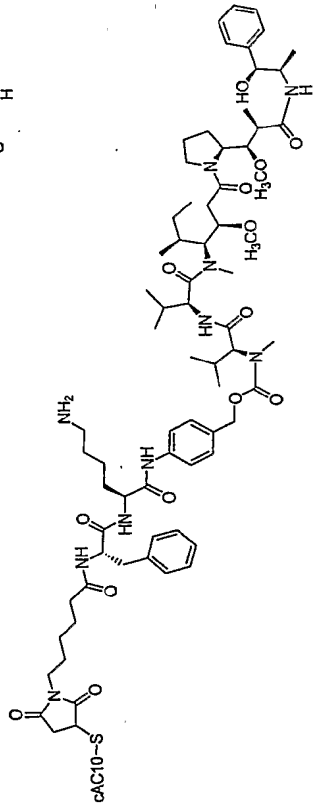

Medium
AC10
HeFi-1
OKT3
AC10+ OKT3
HeFi-1+ OKT3
4 hr
2 hr
46 hr
Id
Annexin V

FIG. 8

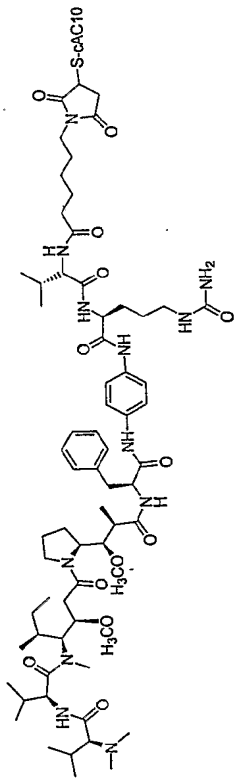
cAC10-vcMMAE



cAC10-fkMMAE



cAC10-vcAEFP



cAC10-fkAEFP

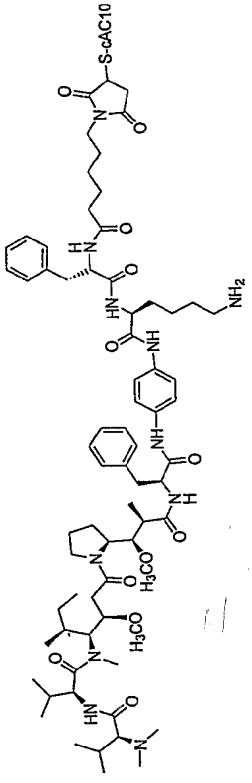


FIG. 9

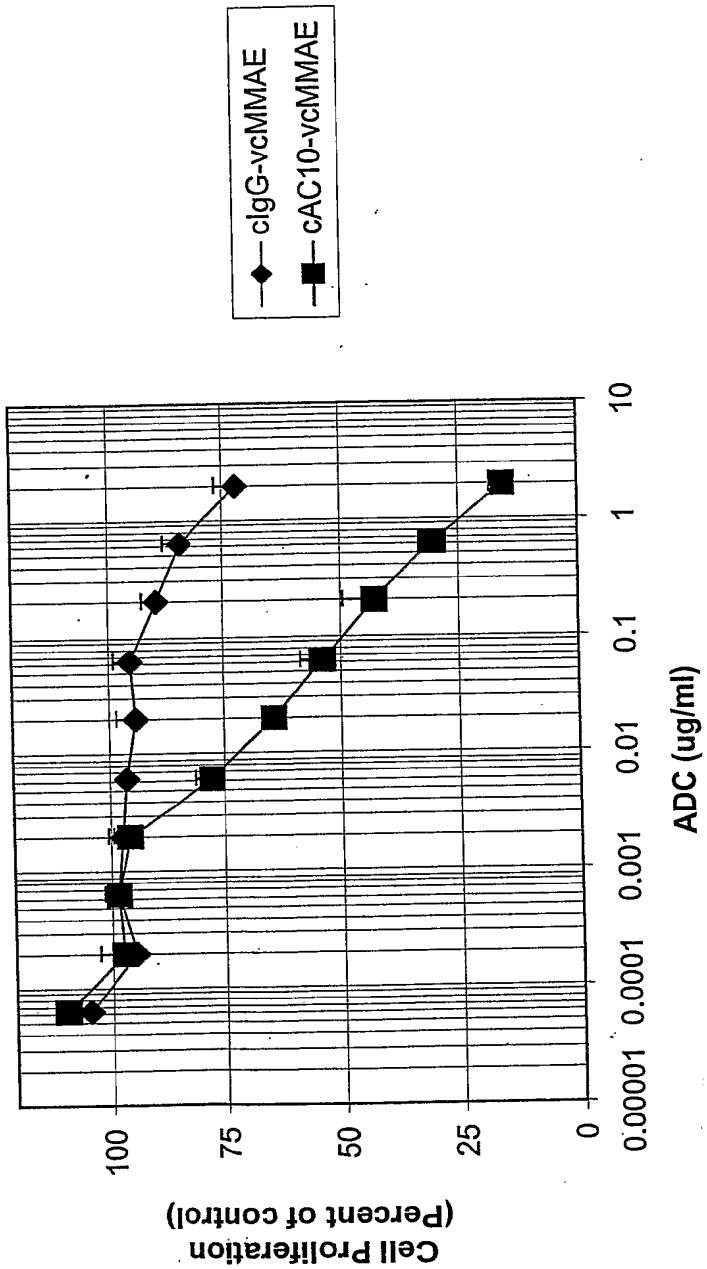

cIgG-vcMMAE
cAC10-vcMMAE
Cell Proliferation
(Percent of control)
100
75
50
25
0
0.00001
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

FIG. 10

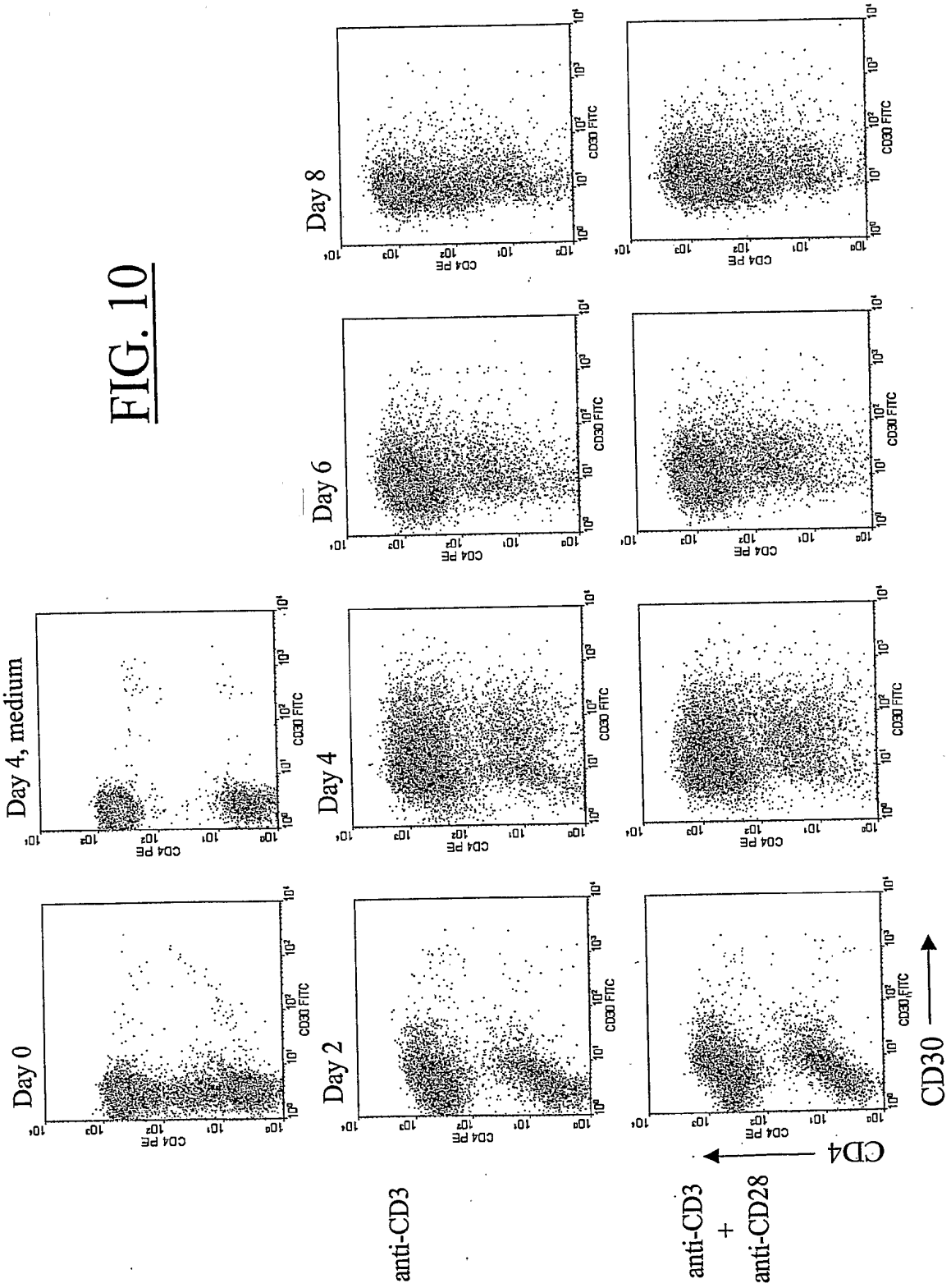
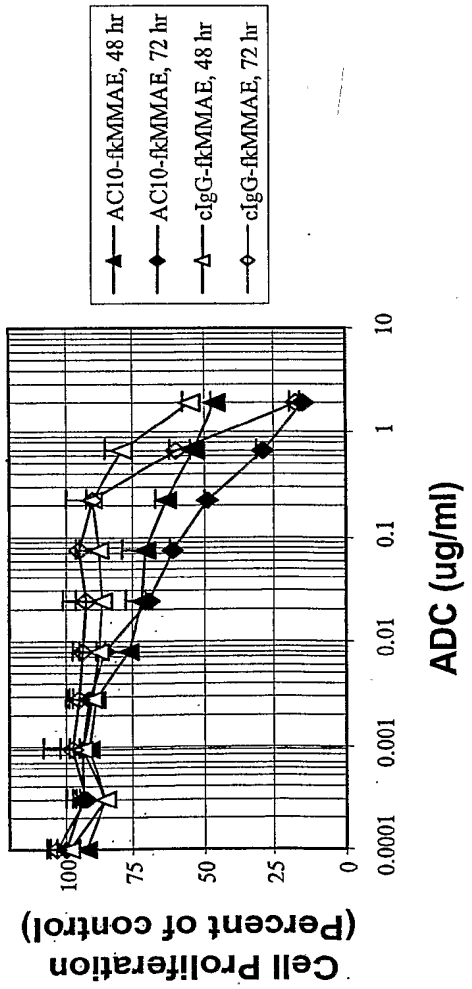

Day 0
Day 2
Day 4
Day 4, medium
Day 6
Day 8
anti-CD3
anti-CD3 + anti-CD28
CD4
CD30
CD4 PE
CD30 FITC

FIG. 11


Antibody-fkMMAE conjugates
AC10-fkMMAE, 48 hr
AC10-fkMMAE, 72 hr
cIgG-fkMMAE, 48 hr
cIgG-fkMMAE, 72 hr
Cell Proliferation (Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

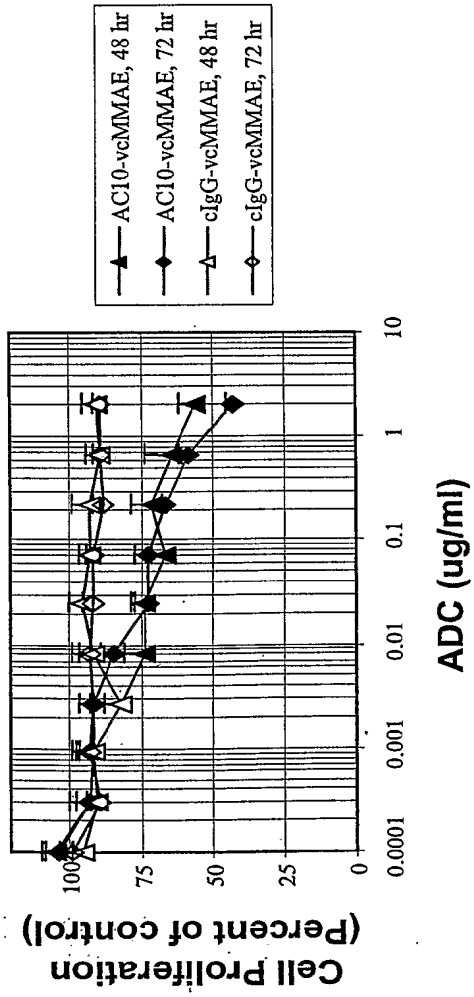

Antibody-vcMMAE conjugates
AC10-vcMMAE, 48 hr
AC10-vcMMAE, 72 hr
cIgG-vcMMAE, 48 hr
cIgG-vcMMAE, 72 hr
Cell Proliferation (Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

FIG. 12

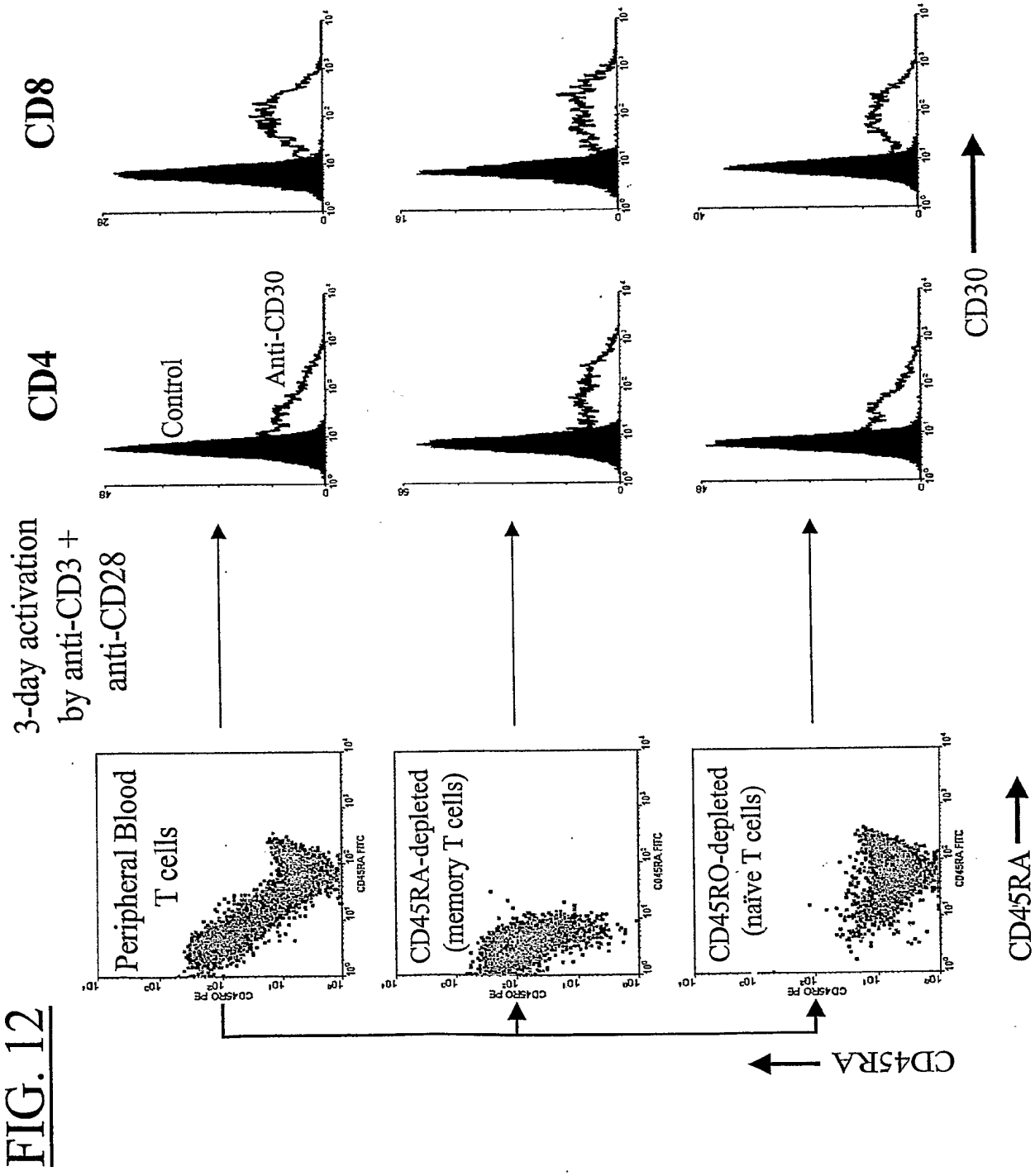

CD8
CD4
Control
Anti-CD30
3-day activation
by anti-CD3 +
anti-CD28
Peripheral Blood
T cells
CD45RA-depleted
(memory T cells)
CD45RO-depleted
(naïve T cells)
CD30
CD45RA
CD45RA
CD45RO

FIG. 13

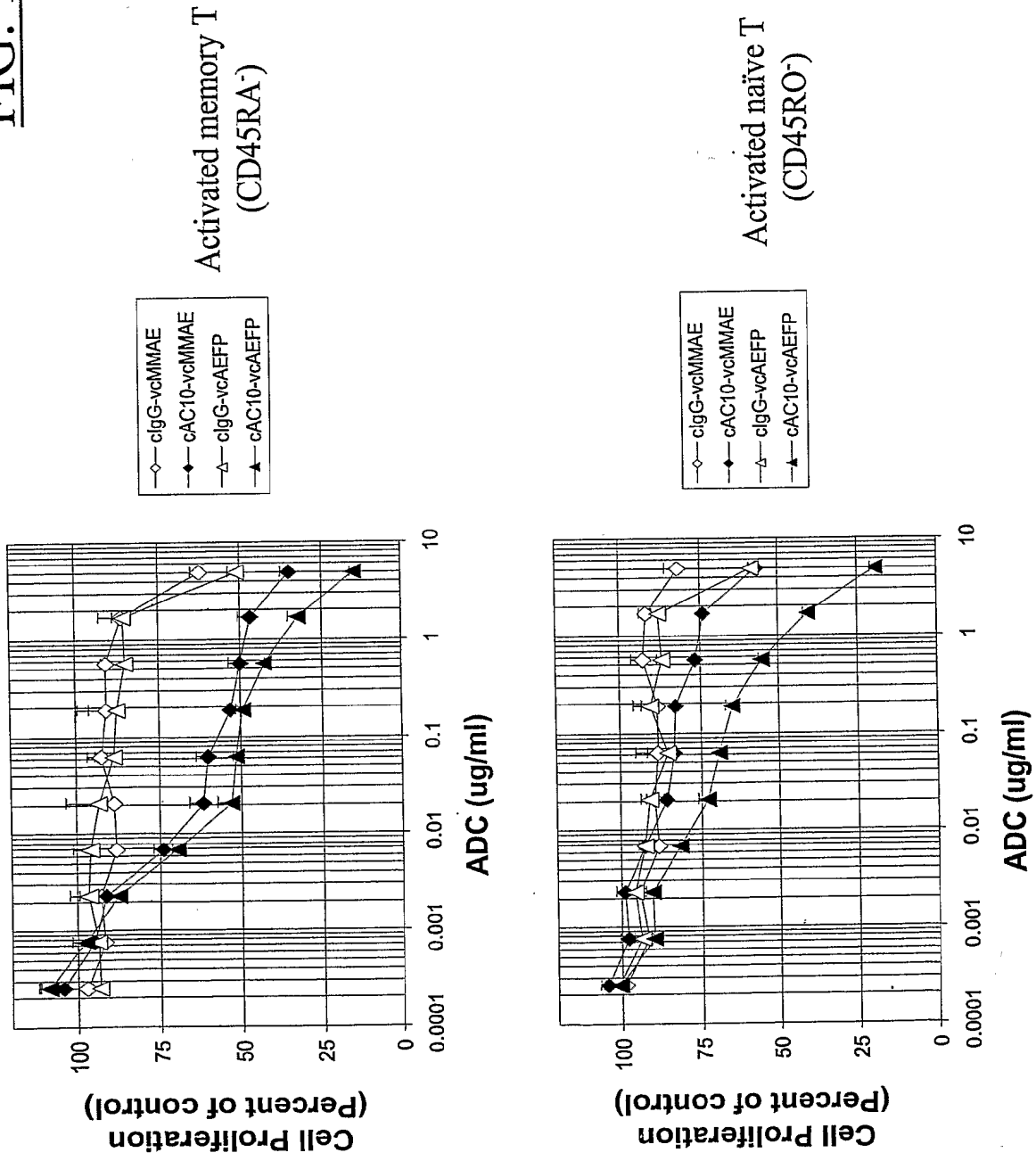

Activated memory T
(CD45RA-)
cIgG-vcMMAE
cAC10-vcMMAE
cIgG-vcAEFP
cAC10-vcAEFP
Cell Proliferation
(Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)
Activated naïve T
(CD45RO-)
cIgG-vcMMAE
cAC10-vcMMAE
cIgG-vcAEFP
cAC10-vcAEFP
Cell Proliferation
(Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

FIG. 14

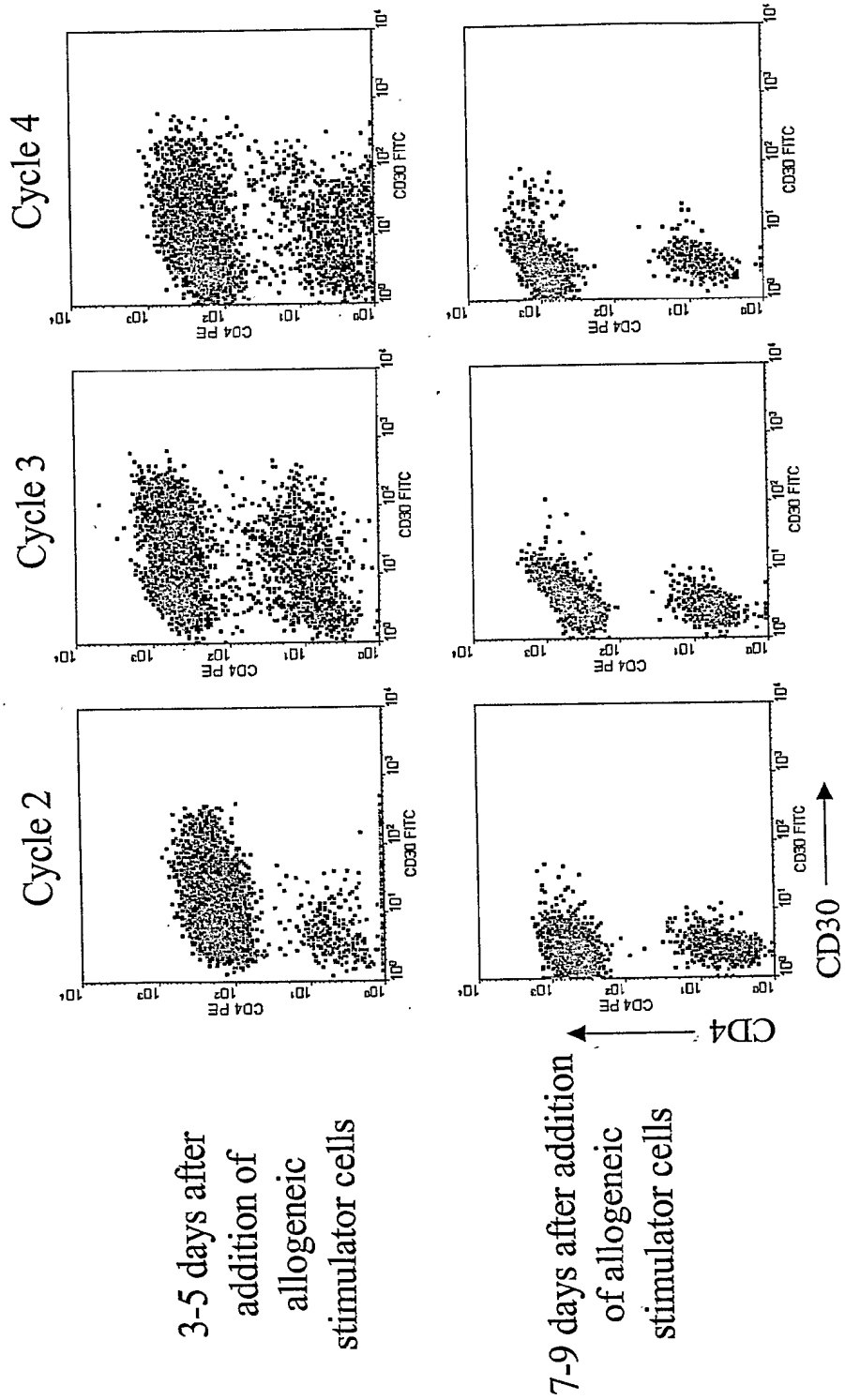

Cycle 2
Cycle 3
Cycle 4
3-5 days after addition of allogeneic stimulator cells
7-9 days after addition of allogeneic stimulator cells
CD4
CD30
CD4 PE
CD30 FITC

FIG. 15

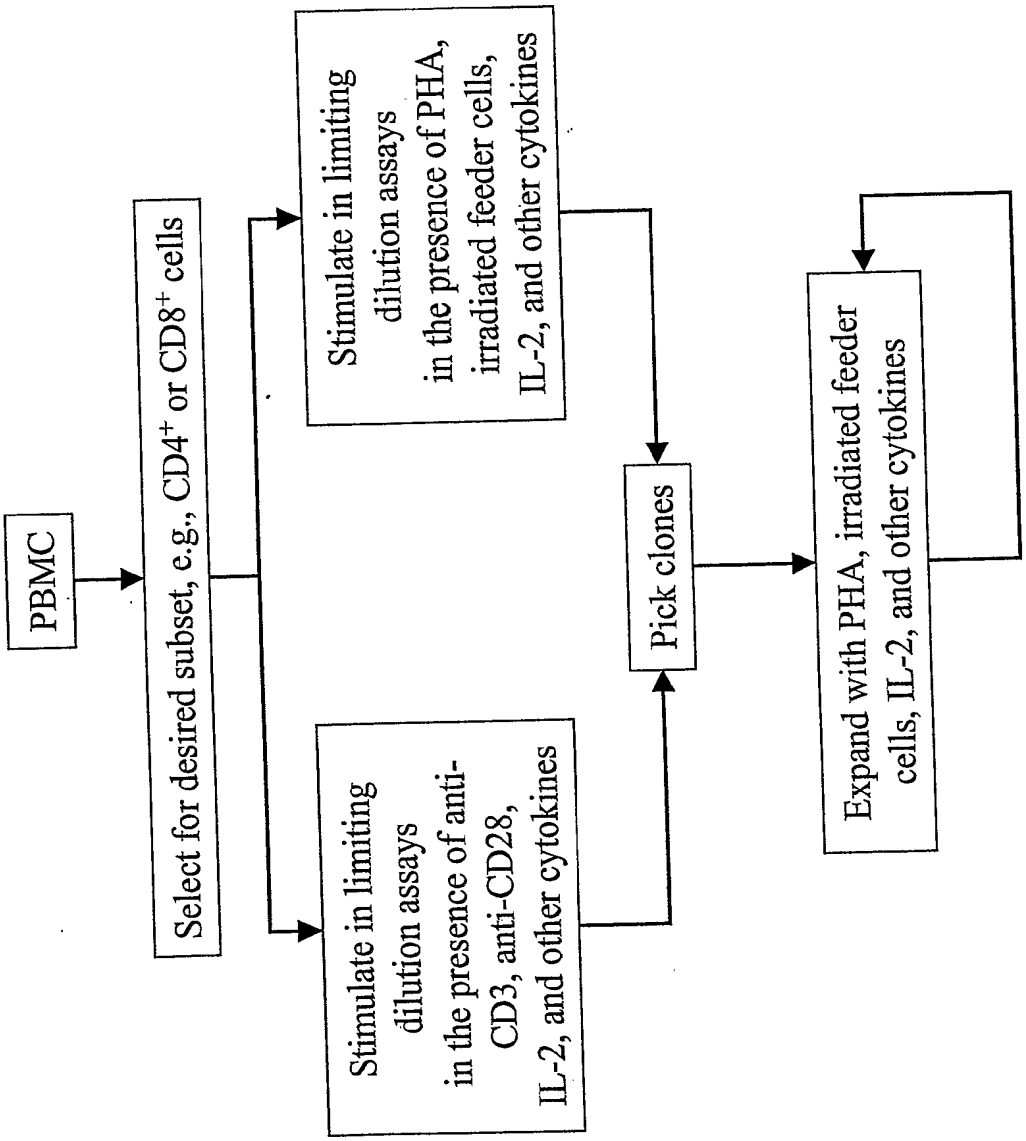
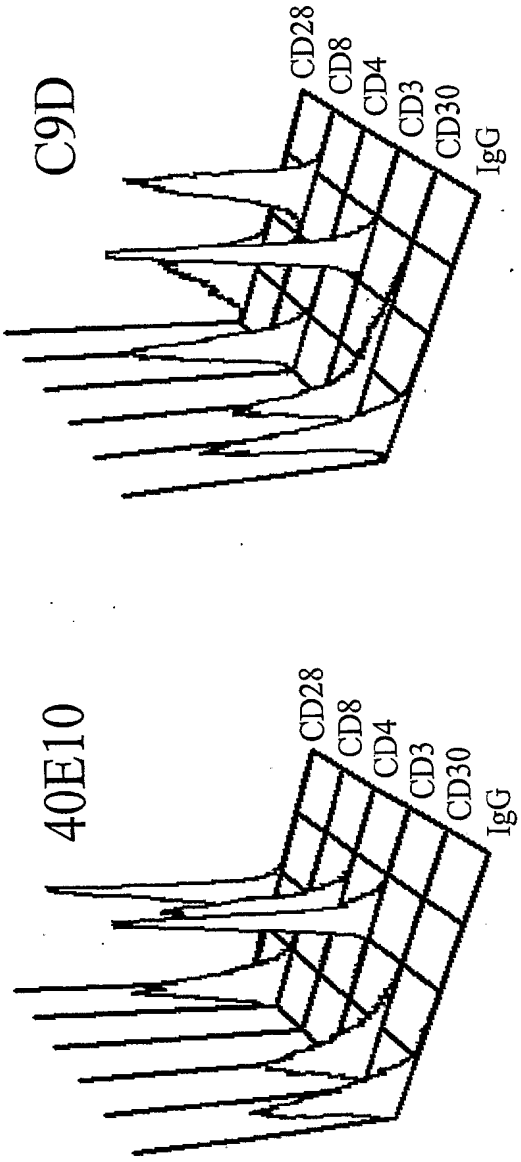
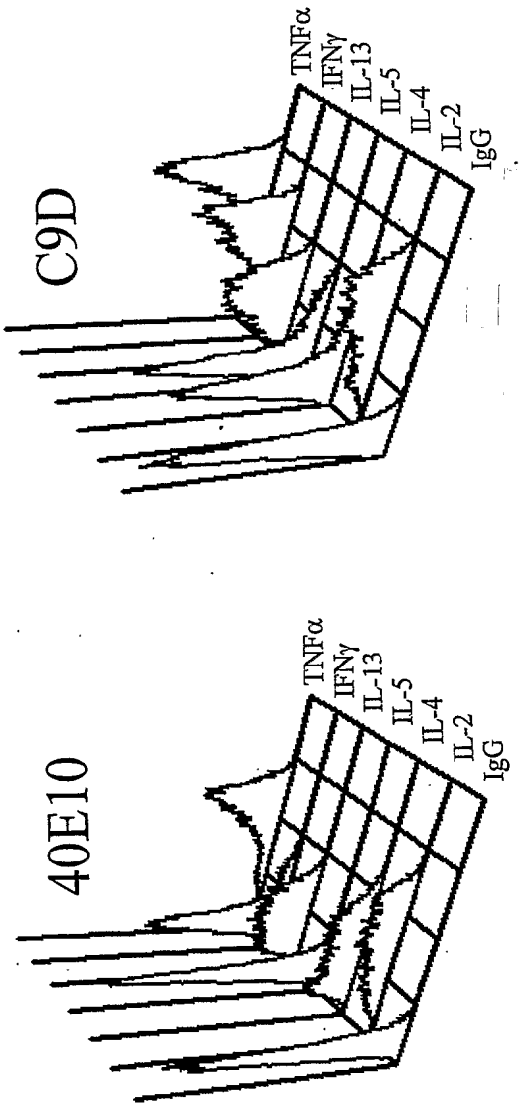

PBMC
Select for desired subset, e.g., CD4+ or CD8+ cells
Stimulate in limiting dilution assays in the presence of anti-CD3, anti-CD28, IL-2, and other cytokines
Stimulate in limiting dilution assays in the presence of PHA, irradiated feeder cells, IL-2, and other cytokines
Pick clones
Expand with PHA, irradiated feeder cells, IL-2, and other cytokines

FIG. 16



	Mean fluorescence intenstiy									
	3.27.2	4.01.1	20G5	40D8	40E10	40H7	C2A	C9D	C5H	C7A
IgG	5	4	6	4	4	4	4	3	3	3
CD30	6	6	10	10	8	8	8	8	6	4
CD3	281	203	209	206	223	224	121	137	135	164
CD4	187	202	168	137	142	150	4	3	4	17
CD8	5	4	5	4	3	4	192	422	98	64
CD28	24	38	37	35	44	46	20	16	10	15

FIG. 17



Mean fluorescence intensity											
	3.27.2	4.01.1	20G5	40D8	40E10	40H7	C1A	C9D	C5H	C7A	
IgG	7	4	4	4	3	3	3	3	3	4	
IL-2	451	556	91	127	88	86	95	170	236	346	
IL-4	24	40	33	23	27	34	27	16	9	13	
IL-5	7	5	10	5	4	6	6	8	26	29	
IL-13	61	64	21	16	14	17	11	25	32	32	
IFNγ	134	165	4	4	7	5	166	138	5	9	
TNFα	998	879	244	253	199	151	425	471	264	239	
	Th0		Th2				Tc0		Tc2		

FIG. 18

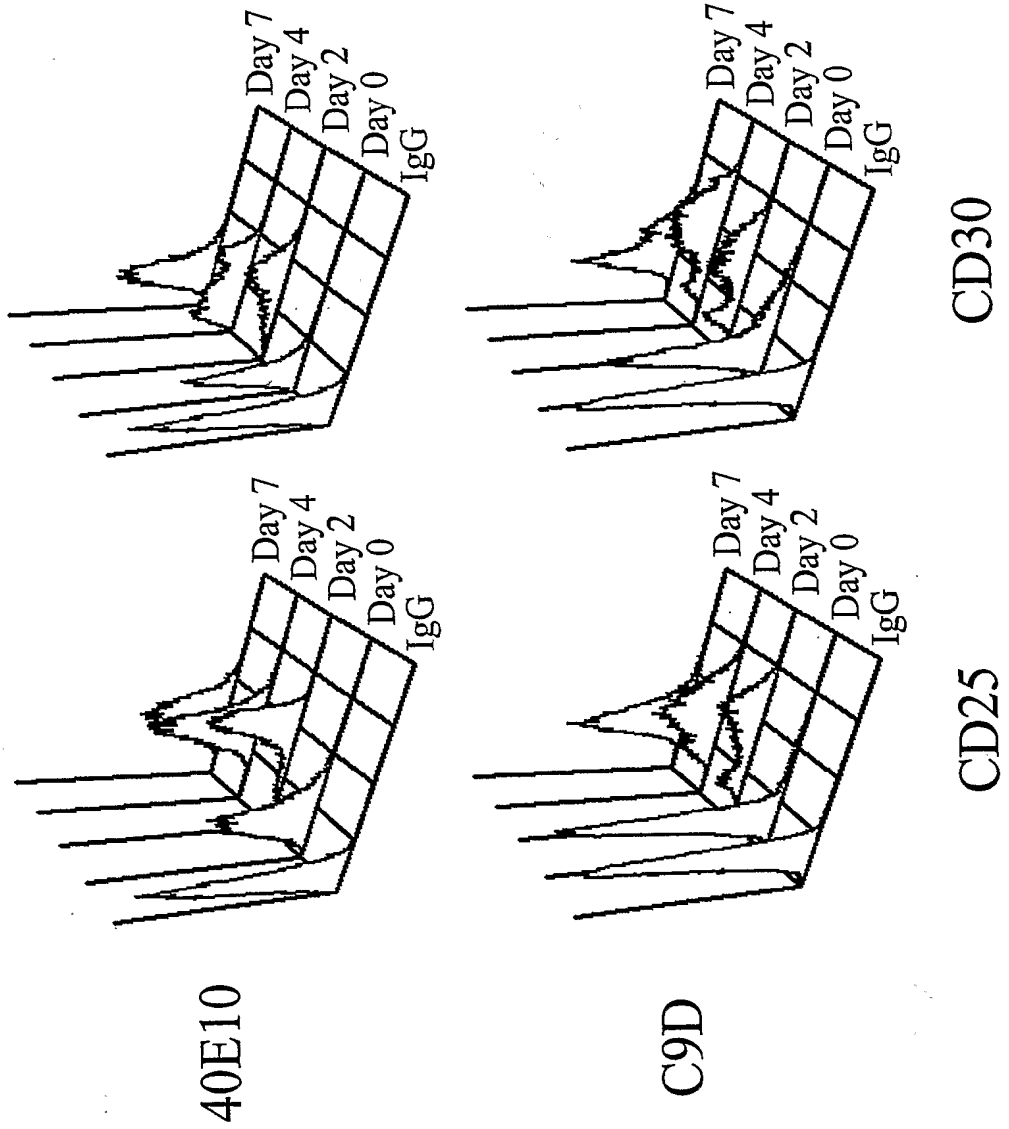
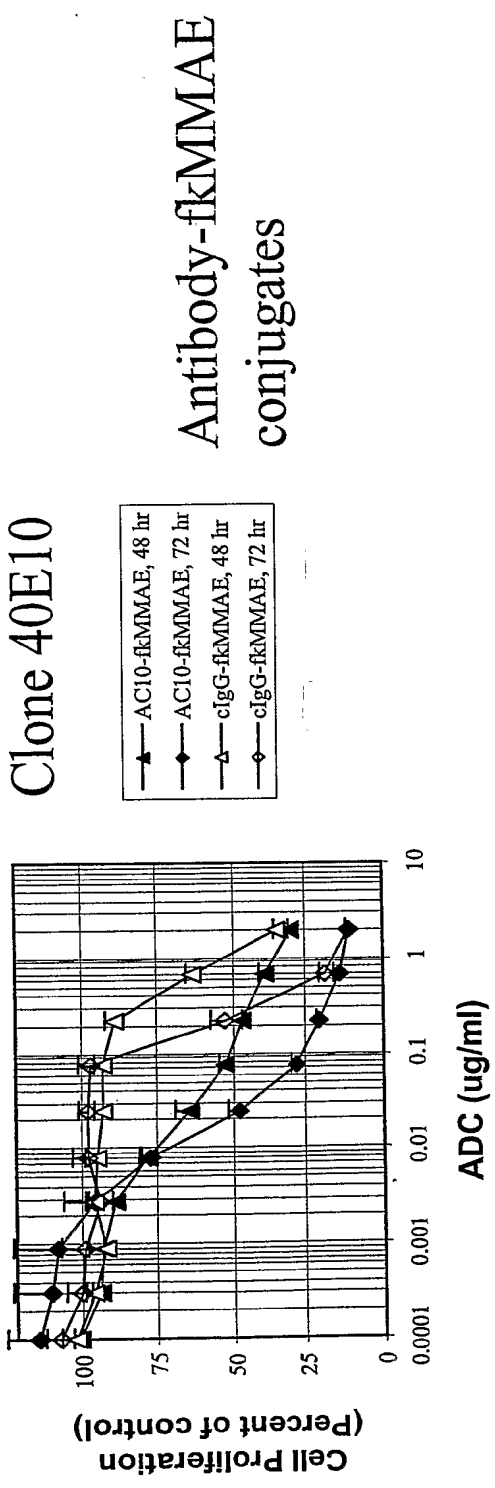


FIG. 19


Clone 40E10
Antibody-fkMMAE conjugates
AC10-fkMMAE, 48 hr
AC10-fkMMAE, 72 hr
cIgG-fkMMAE, 48 hr
cIgG-fkMMAE, 72 hr
Cell Proliferation (Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

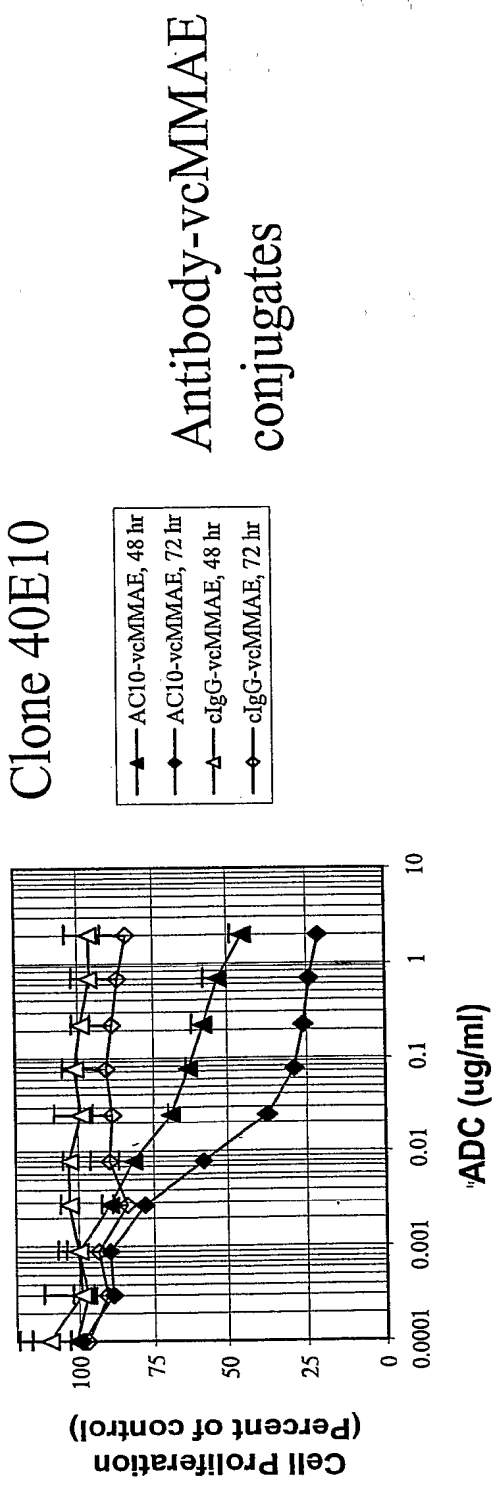

Clone 40E10
Antibody-vcMMAE conjugates
AC10-vcMMAE, 48 hr
AC10-vcMMAE, 72 hr
cIgG-vcMMAE, 48 hr
cIgG-vcMMAE, 72 hr
Cell Proliferation (Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

FIG. 20

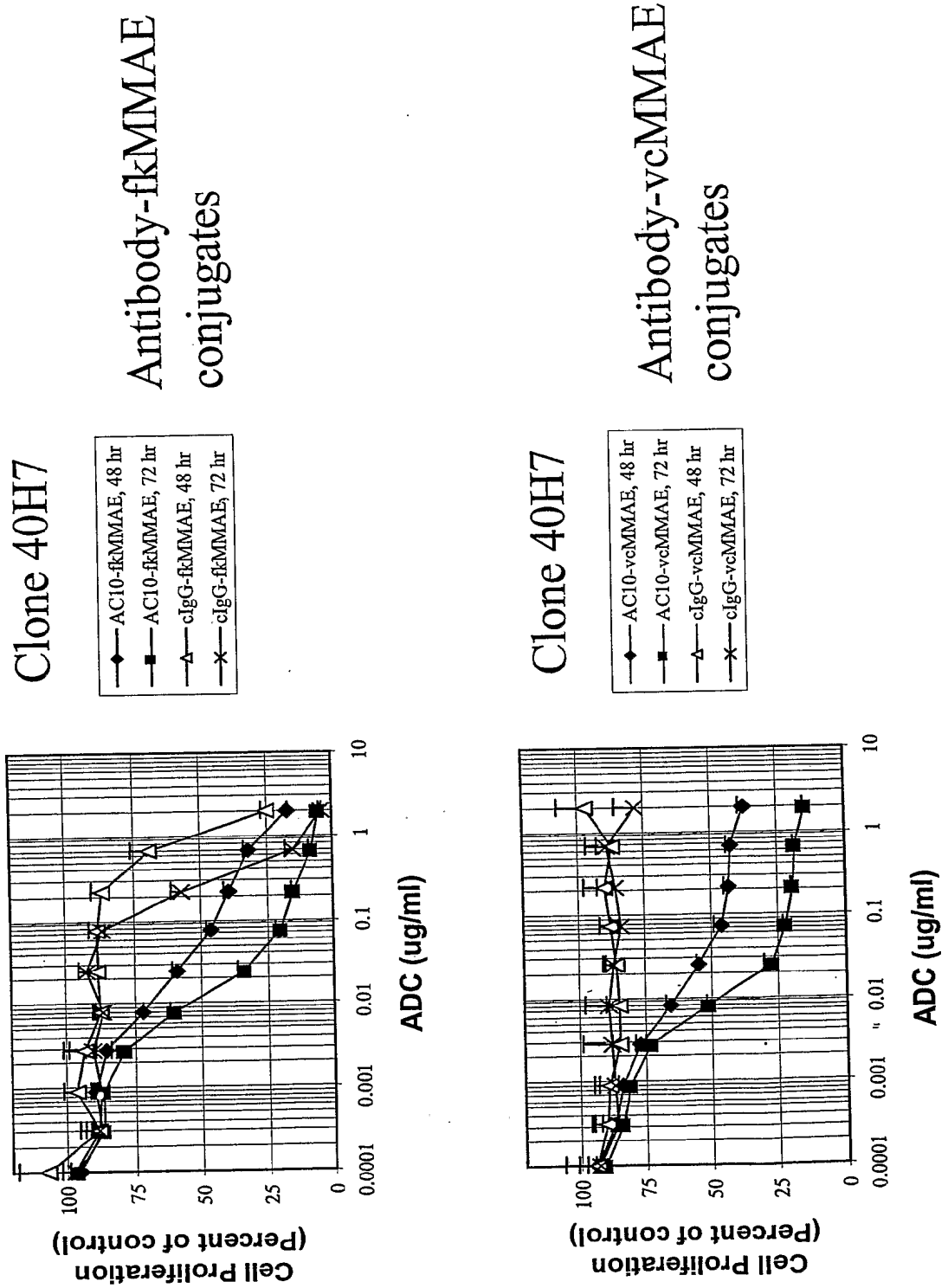
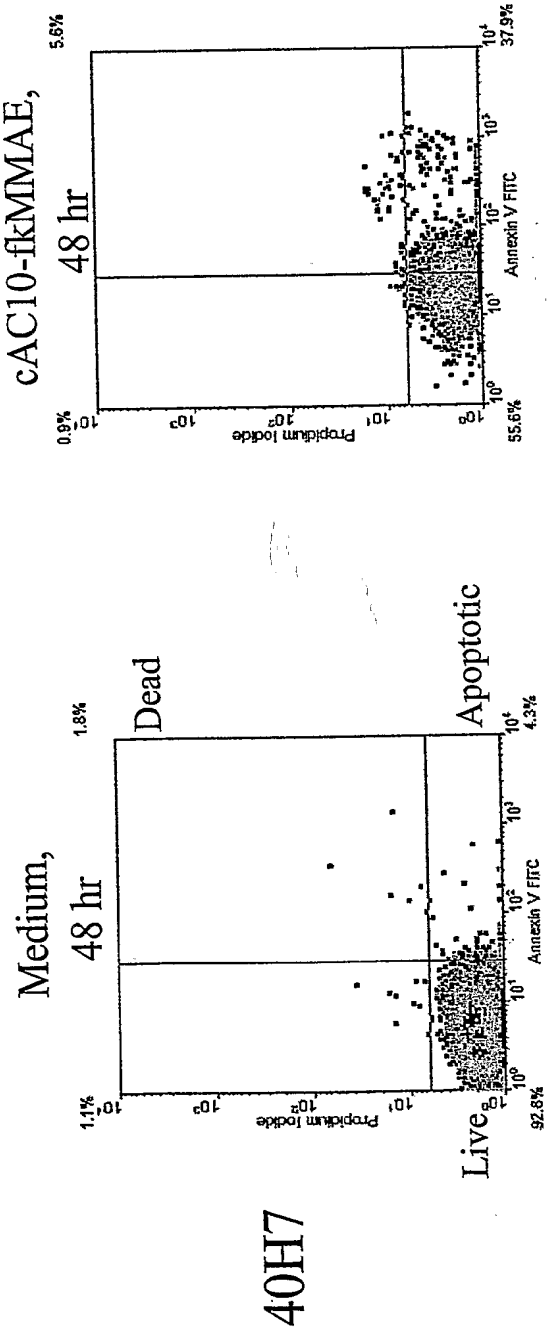

Antibody-fkMMAE conjugates
Clone 40H7
AC10-fkMMAE, 48 hr
AC10-fkMMAE, 72 hr
cIgG-fkMMAE, 48 hr
cIgG-fkMMAE, 72 hr
Cell Proliferation (Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)
Antibody-vcMMAE conjugates
Clone 40H7
AC10-vcMMAE, 48 hr
AC10-vcMMAE, 72 hr
cIgG-vcMMAE, 48 hr
cIgG-vcMMAE, 72 hr
Cell Proliferation (Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

FIG. 21



Percentage of cells in each quadrant

		Medium	cAC10-vcMMAE	cIgG-vcMMAE	cAC10-fkMMAE	cIgG-fkMMAE
40E10, 48 hr	Live	90	73	85	51	68
	Apoptotic	5	22	10	40	26
	Dead	5	5	4	8	5
40H7, 48 hr	Live	93	71	92	56	87
	Apoptotic	4	21	5	38	8
	Dead	2	6	2	6	5

FIG. 22

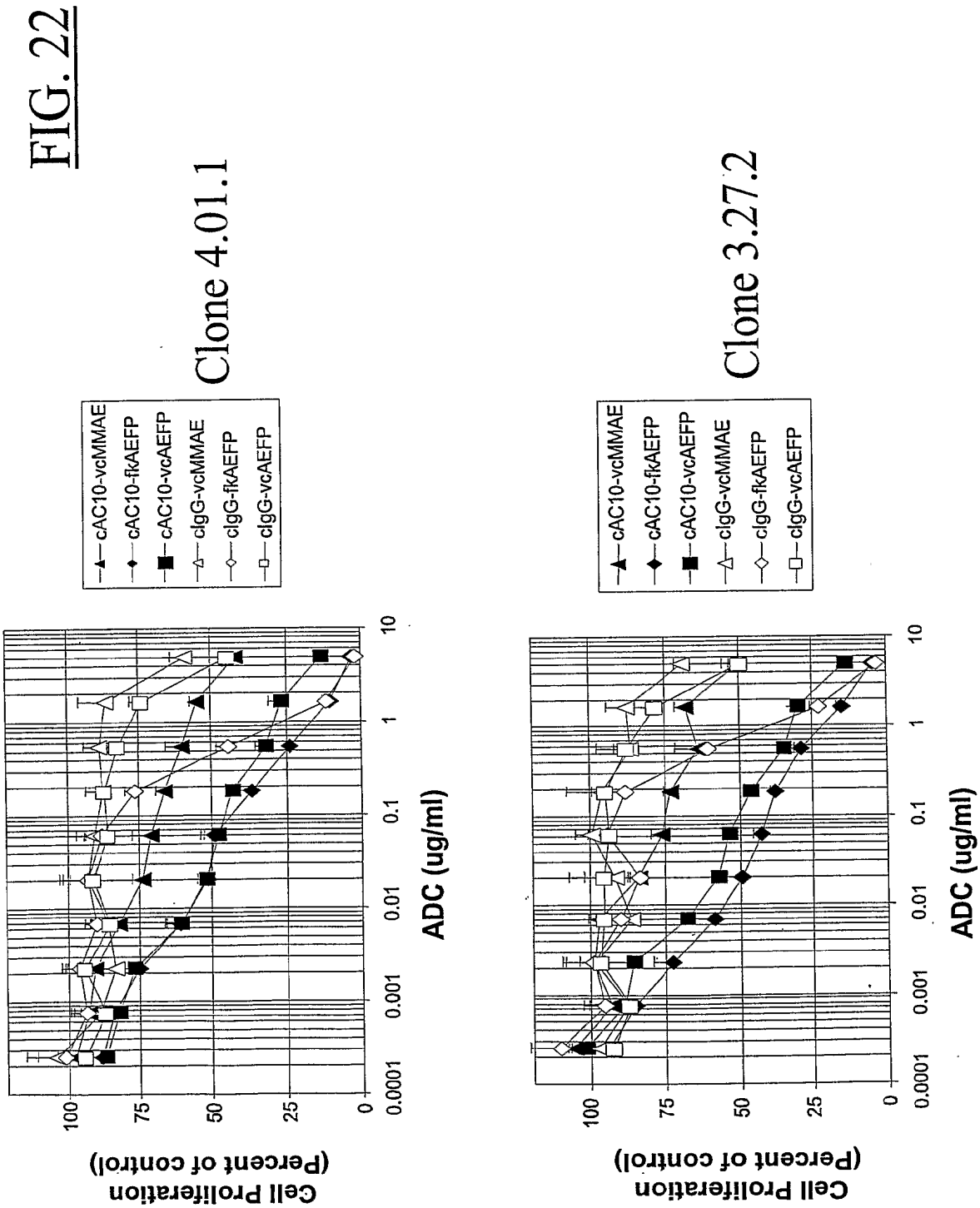

Clone 4.01.1
cAC10-vcMMAE
cAC10-fkAEFP
cAC10-vcAEFP
cIgG-vcMMAE
cIgG-fkAEFP
cIgG-vcAEFP
Cell Proliferation
(Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)
Clone 3.27.2
cAC10-vcMMAE
cAC10-fkAEFP
cAC10-vcAEFP
cIgG-vcMMAE
cIgG-fkAEFP
cIgG-vcAEFP
Cell Proliferation
(Percent of control)
100
75
50
25
0
0.0001
0.001
0.01
0.1
1
10
ADC (ug/ml)

FIG. 23

T cell clone or normal T cells	Phenotype	Cytokine profile	IC50 (ug/ml)	
			cAC10-vcMMAE	cAC10-vcAEFP
3.27.2	CD3, CD4, CD30	IL4, IL-5, IL-13, IFNγ	3.8	0.07
4.01.1	CD3, CD4, CD30	IL4, IL-5, IL-13, IFNγ	2.5	0.01
20G5	CD3, CD4, CD30	IL-4, IL-13	0.006	0.003
40D8	CD3, CD4, CD30	IL-4, IL-13	0.005	0.003
40E10	CD3, CD4, CD30	IL-4, IL-13	0.01	n.d.
40H7	CD3, CD4, CD30	IL-4, IL-13	0.009	n.d.
C1A	CD3, CD8, CD30	IL-4, IL-13, IFNγ	0.63	0.2
C9D	CD3, CD8, CD30	IL-13, IFNγ	0.005	n.d.
C5H	CD3, CD8, CD30	IL-5, IL13	0.037	n.d.
C7A	CD3, CD4/CD8, CD30	IL-5, IL13	0.32	n.d.
Naïve T cells	CD3, CD4, CD8, CD30		>2	1.3
Memory T cells	CD3, CD4, CD8, CD30		1	0.09

n.d. = not determined

SEQUENCE LISTING

110> SEATTLE GENETICS, INC.
Law, Che-Leung
Klussman, Kerry
Wahl, Alan
Senter, Peter
Doronina, Svetlana
Toki, Brian

<120> TREATMENT OF IMMUNOLOGICAL DISORDERS USING ANTI-CD30 ANTIBODIES

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Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp Tyr
            20                  25                  30

tat ata acc tgg gtg aag cag aag cct gga cag gga ctt gag tgg att      144
Tyr Ile Thr Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45

gga tgg att tat cct gga agc ggt aat act aag tac aat gag aag ttc      192
Gly Trp Ile Tyr Pro Gly Ser Gly Asn Thr Lys Tyr Asn Glu Lys Phe
    50                  55                  60

aag ggc aag gcc aca ttg act gta gac aca tcc tcc agc aca gcc ttc      240
Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser Thr Ala Phe
65                  70                  75                  80

atg cag ctc agc agc ctg aca tct gag gac act gct gtc tat ttc tgt      288
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Thr Ala Val Tyr Phe Cys
                85                  90                  95

gcg aac tat ggt aac tac tgg ttt gct tac tgg ggc caa ggg act cag      336
Ala Asn Tyr Gly Asn Tyr Trp Phe Ala Tyr Trp Gly Gln Gly Thr Gln
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            20                  25                  30
Tyr Ile Thr Trp Val Lys Gln Lys Pro Gly Gln Gly Leu Glu Trp Ile
        35                  40                  45
Gly Trp Ile Tyr Pro Gly Ser Gly Asn Thr Lys Tyr Asn Glu Lys Phe
    50                  55                  60
Lys Gly Lys Ala Thr Leu Thr Val Asp Thr Ser Ser Ser Thr Ala Phe
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 December 2005 (01.12.2005)

PCT

(10) International Publication Number
WO 2005/112919 A2

(51) International Patent Classification[7]: A61K 31/403, C07D 43/02

(21) International Application Number:
PCT/US2005/017804

(22) International Filing Date: 19 May 2005 (19.05.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/572,667	19 May 2004 (19.05.2004)	US
60/661,174	9 March 2005 (09.03.2005)	US
60/669,871	8 April 2005 (08.04.2005)	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, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, 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, IS, IT, LT, 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: CHEMICAL LINKERS AND CONJUGATES THEREOF

(57) Abstract: The present disclosure provides drug-ligand conjugates that are potent cytotoxins, wherein the drug is linked to the ligand through either a peptidyl, hydrazine, or disulfide linker. The disclosure is also directed to compositions containing the drug-ligand conjugates, and to methods of treatment using them.

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CHEMICAL LINKERS AND CONJUGATES THEREOF

This application claims the benefit of U.S. Provisional Patent Applications Serial Nos. 60/572,667, 60/661,174, and 60/669,871, which are herein incorporated by reference.

FIELD OF THE INVENTION

The present invention provides linkers that attach to a drug and a ligand and are cleaved in vivo. The linkers are of use in forming prodrugs and conjugates of the cytotoxins of the invention as well as other diagnostic and therapeutic moieties.

BACKGROUND OF THE INVENTION

Many therapeutic agents, particularly those that are especially effective in cancer chemotherapy, often exhibit acute toxicity *in vivo*, especially bone marrow and mucosal toxicity, as well as chronic cardiac and neurological toxicity. Such high toxicity can limit their applications. Development of more and safer specific therapeutic agents, particularly antitumor agents, is desirable for greater effectiveness against tumor cells and a decrease in the number and severity of the side effects of these products (toxicity, destruction of non-tumor cells, etc.). Another difficulty with some existing therapeutic agents is their less than optimal stability in plasma. Addition of functional groups to stabilize these compounds resulted in a significant lowering of the activity. Accordingly, it is desirable to identify ways to stabilize compounds while maintaining acceptable therapeutic activity levels.

The search for more selective cytotoxic agents has been extremely active for many decades, the dose limiting toxicity (i.e. the undesirable activity of the cytotoxins on normal tissues) being one of the major causes of failures in cancer therapy. For example, CC-1065 and the duocarmycins are known to be extremely potent cytotoxins.

CC-1065 was first isolated from *Streptomyces zelensis* in 1981 by the Upjohn Company (Hanka *et al.*, *J. Antibiot.* **31**: 1211 (1978); Martin *et al.*, *J. Antibiot.* **33**: 902 (1980); Martin *et al.*, *J. Antibiot.* **34**: 1119 (1981)) and was found to have potent antitumor and antimicrobial activity both *in vitro* and in experimental animals (Li *et al.*, *Cancer Res.* **42**: 999

(1982)). CC-1065 binds to double-stranded B-DNA within the minor groove (Swenson *et al., Cancer Res.* **42**: 2821 (1982)) with the sequence preference of 5'-d(A/GNTTA)-3' and 5'-d(AAAAA)-3' and alkylates the N3 position of the 3'-adenine by its CPI left-hand unit present in the molecule (Hurley *et al., Science* **226**: 843 (1984)). Despite its potent and broad antitumor activity, CC-1065 cannot be used in humans because it causes delayed death in experimental animals.

Many analogues and derivatives of CC-1065 and the duocarmycins are known in the art. The research into the structure, synthesis and properties of many of the compounds has been reviewed. *See*, for example, Boger *et al., Angew. Chem. Int. Ed. Engl.* **35**: 1438 (1996); and Boger *et al., Chem. Rev.* **97**: 787 (1997).

A group at Kyowa Hakko Kogya Co., Ltd. has prepared a number of CC-1065 derivatives. See, for example, U.S. Pat. No. 5,101, 038; 5,641,780; 5,187,186; 5,070,092; 5,703,080; 5,070,092; 5,641,780; 5,101,038; and 5,084,468; and published PCT application, WO 96/10405 and published European application 0 537 575 A1.

The Upjohn Company (Pharmacia Upjohn) has also been active in preparing derivatives of CC-1065. See, for example, U.S. Patent No. 5,739,350; 4,978,757, 5,332, 837 and 4,912,227.

Research has also focused on the development of new therapeutic agents which are in the form of prodrugs, compounds that are capable of being converted to drugs (active therapeutic compounds) *in vivo* by certain chemical or enzymatic modifications of their structure. For purposes of reducing toxicity, this conversion is preferably confined to the site of action or target tissue rather than the circulatory system or non-target tissue. However, even prodrugs are problematic as many are characterized by a low stability in blood and serum, due to the presence of enzymes that degrade or activate the prodrugs before the prodrugs reach the desired sites within the patient's body.

Bristol-Myers Squibb has described particular lysosomal enzyme-cleavable antitumor drug conjugates. See, for example, U.S. Patent No. 6,214,345. This patent provides an aminobenzyl oxycarbonyl.

Seattle Genetics has published applications U.S. Pat. Appl. 2003/0096743 and U.S. Pat. Appl. 2003/0130189, which describe p-aminobenzylethers in drug delivery agents. The linkers described in these applications are limited to aminobenzyl ether compositions.

Other groups have also described linkers. See for example de Groot *et al., J. Med. Chem.* 42, 5277 (1999); de Groot *et al. J. Org. Chem.* 43, 3093 (2000); de Groot *et al., J. Med. Chem.* 66, 8815, (2001); WO 02/083180; Carl *et al., J. Med. Chem. Lett.* 24, 479, (1981); Dubowchik *et al., Bioorg & Med. Chem. Lett.* 8, 3347 (1998). These linkers include aminobenzyl ether spacer, elongated electronic cascade and cyclization spacer systems, cyclisation eliminations spacers, such as w-amino aminocarbonyls, and a *p* aminobenzy oxycarbonyl linker.

Stability of cytotoxin drugs, including *in vivo* stability, is still an important issue that needs to be addressed. In addition, the toxicity of many compounds makes them less useful, so compositions that will reduce drug toxicity, such as the formation of a cleaveable prodrug, are needed. Therefore, in spite of the advances in the art, there continues to be a need for the development of improved therapeutic agents for the treatment of mammals, and humans in particular, more specifically cytotoxins that exhibit high specificity of action, reduced toxicity, and improved stability in blood relative to known compounds of similar structure. The instant invention addresses those needs.

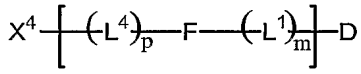
SUMMARY OF THE INVENTION

The present invention relates to drug-ligand conjugates where the drug and ligand are linked through a peptidyl, hydrazine, or disulfide linker. These conjugates are potent cytotoxins that can be selectively delivered to a site of action of interest in an active form and then cleaved to release the active drug. The new linker arms of the invention can be cleaved from the cytotoxic drugs by, for example, enzymatic or reductive means *in vivo*, releasing an active drug moiety from the prodrug derivative. Furthermore, the invention includes conjugates between the linker arms and the cytotoxins of the invention, and conjugates between the linker arms, the cytotoxin and a targeting agent, such as an antibody or a peptide.

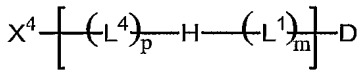
The invention also relates to groups useful for stabilizing therapeutic agents and markers. The stabilizing groups are selected, for example, to limit clearance and metabolism of the therapeutic agent or marker by enzymes that may be present in blood or non-target tissue. The stabilizing groups can serve to block degradation of the agent or marker and may also act in providing other physical characteristics of the agent or marker, for example to increase the solubility of the compound or to decrease the aggregation properties of the compound. The

stabilizing group may also improve the agent or marker's stability during storage in either a formulated or non-formulated form.

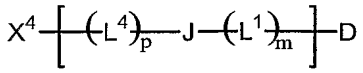
In a first aspect, the invention provides a cytotoxic drug-ligand compound having a structure according to any of Formulas 1-3:



(1)



(2)



(3)

wherein the symbol D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said functional group selected from the group consisting of a primary or secondary amine, hydroxyl, sulfhydryl, carboxyl, aldehyde, and a ketone.

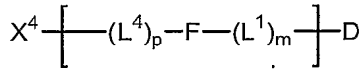
The symbol L^1 represents a self-immolative spacer where m is an integer of 0, 1, 2, 3, 4, 5, or 6.

The symbol X^4 represents a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents.

The symbol L^4 represents a linker member, and p is 0 or 1. L^4 is a moiety that imparts increased solubility or decreased aggregation properties to the conjugates. Examples of L^4 moieties include substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroalkyl, or unsubstituted heteroalkyl, any of which may be straight, branched, or cyclic, a positively or negatively charged amino acid polymer, such as polylysine or polyargenine, or other polymers such as polyethylene glycol.

The symbols F, H, and J represent linkers, as described further herein.

In one embodiment, the invention pertains to peptide linker conjugate of the structure:



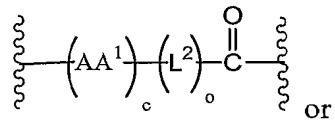
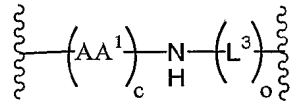
wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said functional group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:


or


wherein

AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^2 is a self-immolative linker;

L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group; wherein if L^3 is present, m is 0 and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D;

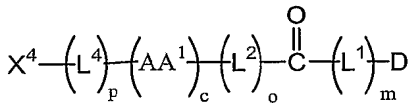
o is 0 or 1;

L^4 is a linker member, wherein L^4 does not comprise a carboxylic acyl group directly attached to the N-terminus of $(AA^1)_c$;

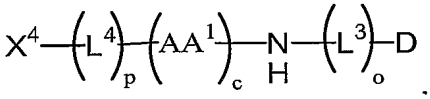
p is 0 or 1; and

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents.

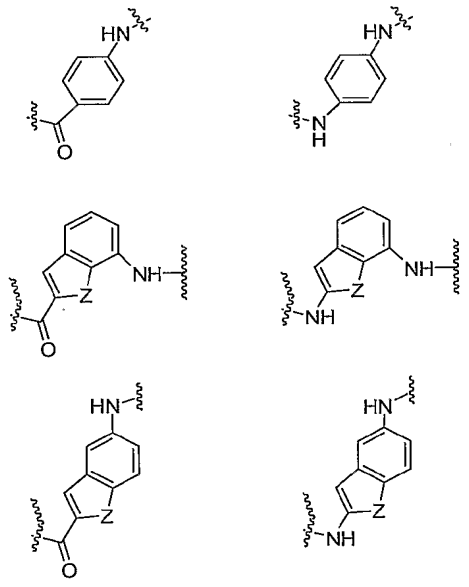
In one embodiment, the peptide linker conjugate comprises the following structure:



In another embodiment, the peptide linker conjugate comprises the following structure:



In a preferred embodiment, L^3 comprises an aromatic group. For example, L^3 can comprise a benzoic acid group, an aniline group, or an indole group. Non-limiting examples of $-L^3-NH-$ include structures selected from the following group:

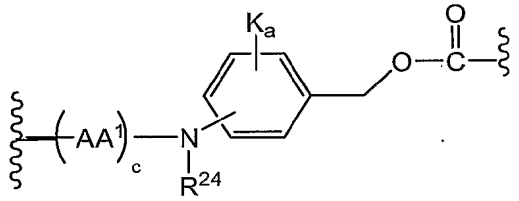


wherein Z is a member selected from O, S and NR^{23}, and

wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

In preferred embodiments of the peptide linker, $(AA^1)_c$ is a peptide sequence cleavable by a protease expressed in tumor tissue. A preferred protease is a lysosomal protease. In preferred embodiments, c is an integer from 2 to 6, or c is 2, 3 or 4. In certain embodiments, the amino acid in $(AA^1)_c$ located closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. In preferred embodiments, $(AA^1)_c$ is a peptide sequence selected from the group consisting of Val-Cit, Val-Lys, Phe-Lys, Lys-Lys, Ala-Lys, Phe-Cit, Leu-Cit, Ile-Cit, Trp, Cit, Phe-Ala, Phe-N^9-tosyl-Arg, Phe-N^9-nitro-Arg, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Leu-Ala-Leu, Ile-Ala-Leu, Val-Ala-Val, Ala-Leu-Ala-Leu (SEQ ID NO: 1), β-Ala-Leu-Ala-Leu (SEQ ID NO: 2) and Gly-Phe-Leu-Gly (SEQ ID NO: 3). In particularly preferred embodiments, $(AA^1)_c$ is Val-Cit or Val-Lys.

In some preferred embodiments, the peptide linker, F, comprises the structure:



wherein

R^{24} is selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl;

Each K is a member independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}

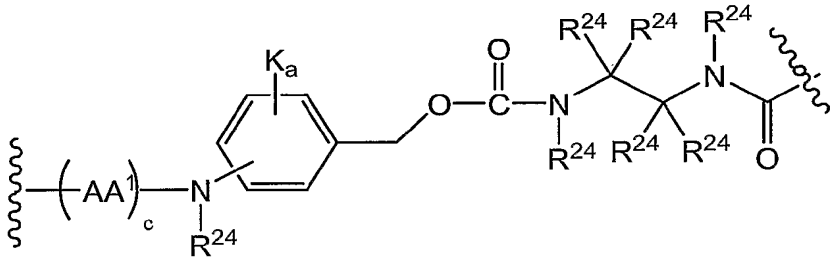
wherein

R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl,

substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl; and

a is an integer of 0,1, 2, 3, or 4.

In other preferred embodiments, -F-$(L^1)_m$- comprises the structure:



wherein

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl.

In another aspect, the invention pertains to hydrazine linker conjugates of the structure:

$$X^4\text{——}(L^4)_p\text{—H—}(L^1)_m\text{——D}$$

wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said function group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

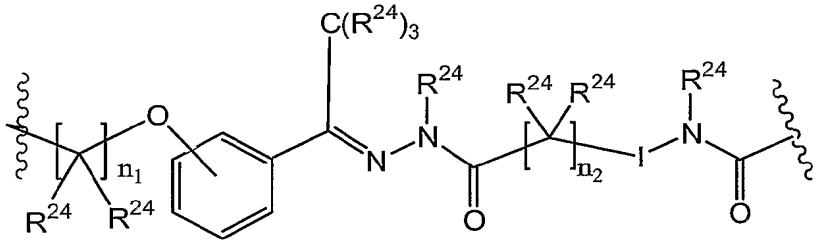
m is an integer selected from 0, 1, 2, 3, 4, 5, or 6;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents;

L^4 is a linker member;

p is 0 or 1;

H is a linker comprising the structure:



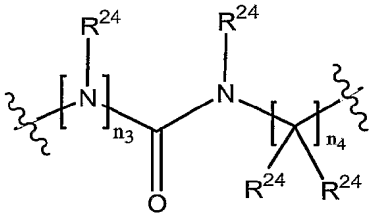
wherein

n_1 is an integer from 1 – 10;

n_2 is 0, 1, or 2;

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl; and

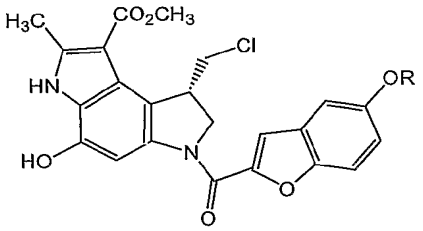
I is either a bond or:



wherein n_3 is 0 or 1 with the proviso that when n_3 is 0, n_2 is not 0; and

n_4 is 1, 2, or 3,

wherein when I is a bond, n_1 is 3 and n_2 is 1, D can not be



where R is Me or CH_2- CH_2-NMe_2.

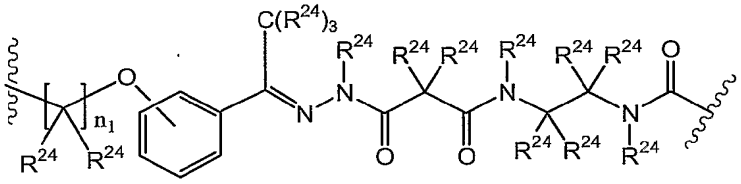
In some preferred embodiments, the substitution on the phenyl ring is a para substitution. In some preferred embodiments, n_1 is 2, 3, or 4 or n_1 is 3 or n_2 is 1.

In certain embodiments, I is a bond. In other embodiments, n_3 is 0 and n_4 is 2.

In various aspects, the invention provides hydrazine linkers, H, that can form a 6-membered self immolative linker upon cleavage, or two 5-membered self immolative linkers upon cleavage, or a single 5-membered self immolative linker upon cleavage, or a single 7-membered self immolative linker upon cleavage, or a 5-membered self immolative linker and a 6-membered self immolative linker upon cleavage.

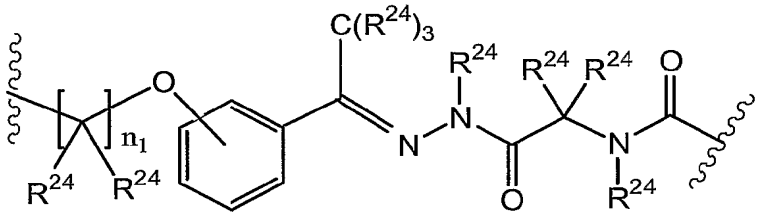
In a preferred embodiment, H comprises a geminal dimethyl substitution.

In a preferred embodiment, H comprises the structure:



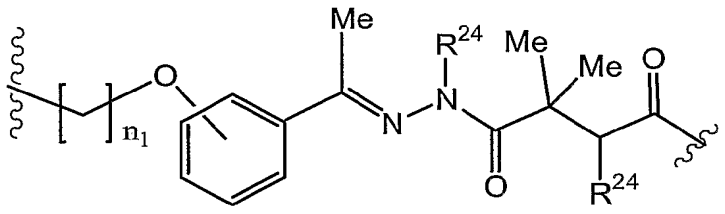
Preferably, n_1 is 2, 3, or 4, more preferably n_1 is 3. Preferably, each R_{24} is independently selected from CH_3 and H. In certain preferred embodiments, each R_{24} is H.

In another preferred embodiment, H comprises the structure:



Preferably, n_1 is 3. Preferably, each R_{24} is independently selected from CH_3 and H.

In yet other preferred embodiments, H comprises the structure:



Preferably, each R^{24} independently an H or a substituted or unsubstituted alkyl.

In another aspect, the invention pertains to hydrazine linker conjugates of the structure:

$$X^4\text{——}(L^4)_p\text{—H—}(L^1)_m\text{——D}$$

wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said function group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

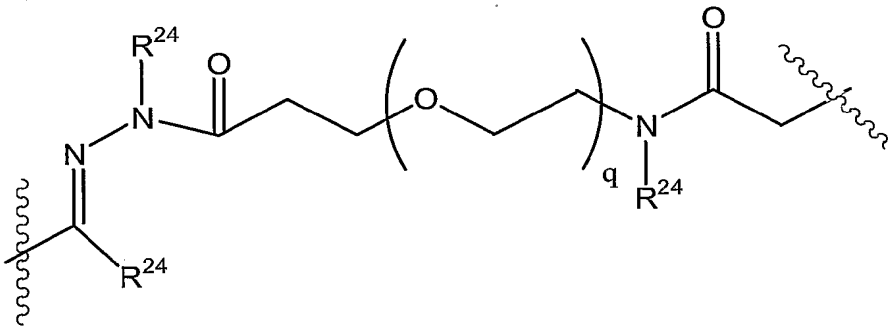
m is an integer selected from 0, 1, 2, 3, 4, 5, or 6;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents;

L^4 is a linker member;

p is 0 or 1; and

H comprises the structure:



where q is 0, 1,2, 3, 4, 5, or 6; and

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl.

In yet another aspect, the invention pertains to disulfide linker conjugates of the structure:

$$X^4\text{—}\left[\text{—}(L^4)_p\text{—J—}(L^1)_m\text{—}\right]\text{—D}$$

wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said function group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

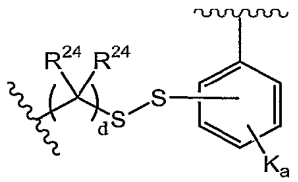
m is an integer selected from 0, 1, 2, 3, 4, 5, or 6;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents;

L^4 is a linker member;

P is 0 or 1;

J is a linker comprising the structure:



wherein

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl;

each K is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}

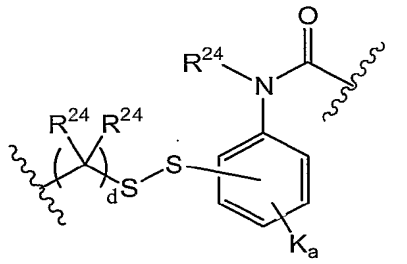
wherein

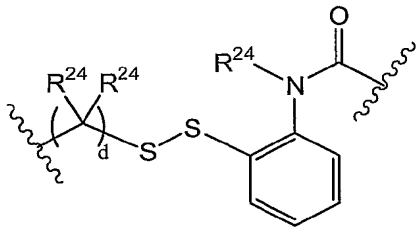
R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl and unsubstituted heterocycloalkyl;

a is an integer of 0,1, 2, 3, or 4; and

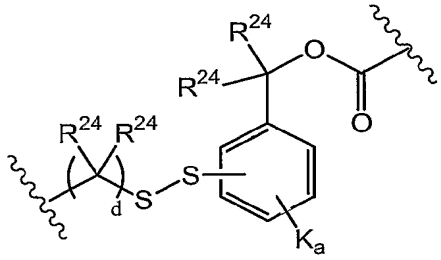
d is an integer of 0, 1, 2, 3, 4, 5, or 6.

In various embodiments, J can comprise one of the following structures:

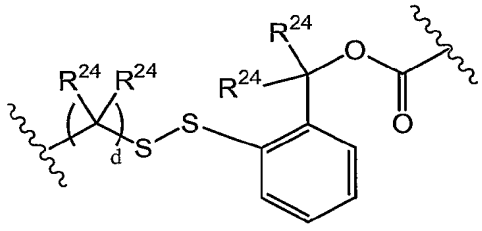




wherein d is 1 or 2;



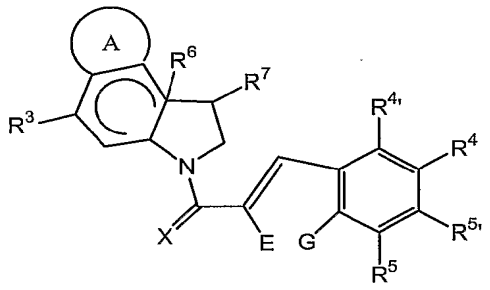
or



In all of the foregoing linker conjugates, D preferably is a cytotoxic drug. In preferred embodiments, D has a chemically reactive function group selected from the group consisting of a primary or secondary amine, hydroxyl, sulfhydryl and carboxyl. Non-limiting examples of preferred drugs, D, include duocarmycins and duocarmycin analogs and derivatives, CC-1065,

CBI-based duocarmycin analogues, MCBI-based duocarmycin analogues, CCBI-based duocarmycin analogues, doxorubicin, doxorubicin conjugates, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatins, dolestatin-10, combretastatin, calicheamicin, maytansine, maytansine analogues, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones, Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin, daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin, podophyllotoxin derivatives, etoposide, etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 -acetyl spermidine and camptothecin.

In a preferred embodiment, D is a duocarmycin analog or derivative that comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

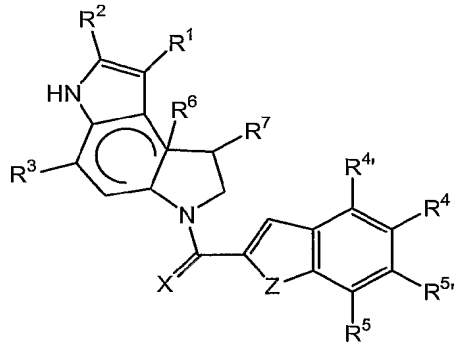
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F, H, or J.

In a preferred embodiment, D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from group consisting of substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, and OR^9

in which

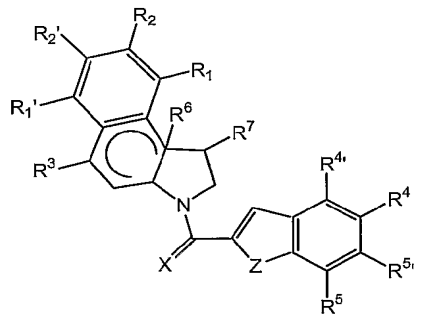
R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^2 is H, substituted alkyl or unsubstituted lower alkyl;

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F, H, or J.

In a preferred embodiment of the above, R^2 is an unsubstituted lower alkyl.

In another preferred embodiment, D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and

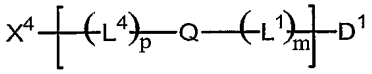
$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F, H, or J.

In all of the foregoing linker conjugate structures, L^4 preferably comprises a non-cyclic moiety. L^4 preferably increases solubility of the compound as compared to the compound

lacking L^4 and/or L^4 decreases aggregation of the compound as compared to the compound lacking L^4. In a preferred embodiment, L^4 comprises a polyethylene glycol moiety. The polyethylene glycol moiety can contain, for example, 3-12 repeat units, or 2-6 repeat units or, more preferably, 4 repeat units.

In yet another aspect, the invention provides a cytotoxic drug-ligand compound having a structure according to the following formula:



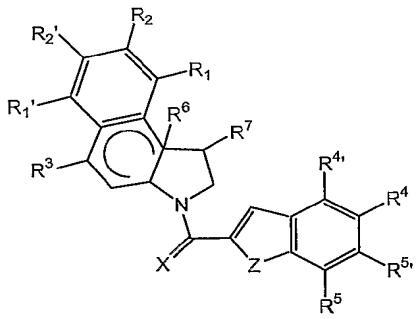
wherein the symbol L^1 represents a self-immolative spacer where m is an integer of 0, 1, 2, 3, 4, 5, or 6.

The symbol X^4 represents a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents.

The symbol L^4 represents a linker member, and p is 0 or 1. L^4 is a moiety that imparts increased solubility or decreased aggregation properties to the conjugates. Examples of L^4 moieties include substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroalkyl, or unsubstituted heteroalkyl, any of which may be straight, branched, or cyclic, a positively or negatively charged amino acid polymer, such as polylysine or polyargenine, or other polymers such as polyethylene glycol.

The symbol Q represent any cleavable linker including, but not limited to, any of the peptidyl, hydrozone, and disulfide linkers described herein. Cleavable linkers include those that can be selectively cleaved by a chemical or biological process and upon cleavage separate the drug, D^1, from X^4.

The symbol D^1 represents a drug having the following formula:



wherein X and Z are members independently selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8,

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$,

wherein R^8 is a member selected from NR^9R^{10} and OR^9 and R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy;

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

R^3 is a member selected from the group consisting of SR^{11}, NHR^{11} and OR^{11}, wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$, in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of R^{11}, R^{12}, and R^{13} links said drug to L^1, if present, or to Q,

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

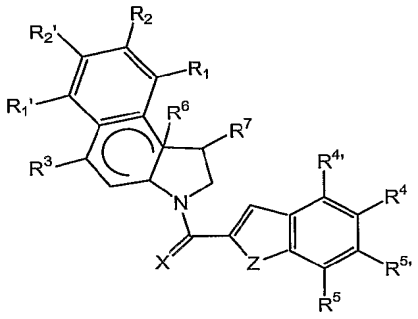
R^7 is CH_2-X^1 or $-CH_2-$ joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group,

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nNR^{24}R^{25}$ wherein n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

and R^{24} and R^{25} are independently selected from unsubstituted alkyl, and wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ is $O(CH_2)_nNR^{24}R^{25}$.

Yet another embodiment is a compound having a structure according to Formula 1:



(1)

wherein X and Z are independently selected from O, S and NR^{23} wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8,

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$,

each R^8 is a member independently selected from NR^9R^{10} and OR^9 and R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, substituted or unsubstituted lower alkyl, unsubstituted heteroalkyl, cyano, or alkoxy;

$R^{2'}$ is H, substituted or unsubstituted lower alkyl, or unsubstituted heteroalkyl,

R^3 is a member selected from the group consisting of SR^{11}, NHR^{11} and OR^{11}, wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$, in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, or R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2-$ joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group,

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nNR^{24}R^{25}$ wherein n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

and R^{24} and R^{25} are independently selected from unsubstituted alkyl, and wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ is $O(CH_2)_nNR^{24}R^{25}$.

In yet another aspect, the invention pertains to pharmaceutical formulations. Such formulations typically comprise a conjugate compound of the invention and a pharmaceutically acceptable carrier.

In still a further aspect, the invention pertains to methods of using the conjugate compounds of the invention. For example, the invention provides a method of killing a cell, wherein a conjugate compound of the invention is administered to the cell an amount sufficient to kill the cell. In a preferred embodiment, the cell is a tumor cell. In another embodiment, the invention provides a method of retarding or stopping the growth of a tumor in a mammalian subject, wherein a conjugate compound of the invention is administered to the subject an amount sufficient to retard or stop growth of the tumor.

Other aspects, advantages and objects of the invention will be apparent from review of the detailed description below.

DETAILED DESCRIPTION OF THE INVENTION

Abbreviations

As used herein, "Ala," refers to alanine.

"Boc," refers to *t*-butyloxycarbonyl.

"CPI," refers to cyclopropapyrroloindole.

"Cbz," is carbobenzoxy.

As used herein, "DCM," refers to dichloromethane.

"DDQ," refers to 2,3-dichloro-5,6-dicyano-1,4-benzoquinone.

DIPEA is diisopropylethalamine

"DMDA" is N,N'-dimethylethylene diamine

"RBF" is a round bottom flask

"DMF" is N,B-dimethylformamide

"HATU" is N-[[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]pyridin-1-yl]methylene]-N-methylmethanaminium hexafluorophosphate N-oxide

As used herein, the symbol "E," represents an enzymatically cleaveable group.

"EDCI" is 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide.

As used herein, "FMOC," refers to 9-fluorenylmethyloxycarbonyl.

"FMOC" irefers to 9-fluorenylmethoxycarbonyl.

"HOAt" is 7-Aza-1-hydroxybenzotriazole.

"Leu" is leucine.

"PABA" refers to *para*-aminobenzoic acid.

PEG refers to polyethylene glycol

"PMB," refers to *para*-methoxybenzyl.

"TBAF," refers to tetrabutylammonium fluoride.

The abbreviation "TBSO," refers to *t*-butyldimethylsilyl ether.

As used herein, "TEA," refers to triethylamine.

"TFA," refers to trifluororoacetic acid.

The symbol "Q" refers to a therapeutic agent, diagnostic agent or detectable label.

Definitions

Unless defined otherwise, all technical and scientific terms used herein generally have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in cell culture, molecular genetics, organic chemistry and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for nucleic acid and peptide synthesis. Generally, enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (*see generally*, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference), which are provided throughout this document. The nomenclature used herein and the laboratory procedures in analytical chemistry, and organic synthetic described below are those well known and commonly employed in the art. Standard techniques, or modifications thereof, are used for chemical syntheses and chemical analyses.

The term "therapeutic agent" is intended to mean a compound that, when present in a therapeutically effective amount, produces a desired therapeutic effect on a mammal. For

treating carcinomas, it is desirable that the therapeutic agent also be capable of entering the target cell.

The term "cytotoxin" is intended to mean a therapeutic agent having the desired effect of being cytotoxic to cancer cells. Cytotoxic means that the agent arrests the growth of, or kills the cells. Exemplary cytotoxins include, by way of example and not limitation, combretastatins, duocarmycins, the CC-1065 anti-tumor antibiotics, anthracyclines, and related compounds. Other cytotoxins include mycotoxins, ricin and its analogues, calicheamycins, doxirubicin and maytansinoids.

The term "prodrug" and the term "drug conjugate" are used herein interchangeably. Both refer to a compound that is relatively innocuous to cells while still in the conjugated form but which is selectively degraded to a pharmacologically active form by conditions, e.g., enzymes, located within or in the proximity of target cells.

The term "marker" is intended to mean a compound useful in the characterization of tumors or other medical condition, for example, diagnosis, progression of a tumor, and assay of the factors secreted by tumor cells. Markers are considered a subset of "diagnostic agents."

The term "selective" as used in connection with enzymatic cleavage means that the rate of rate of cleavage of the linker moiety is greater than the rate of cleavage of a peptide having a random sequence of amino acids.

The terms "targeting group" and "targeting agent" are intended to mean a moiety that is (1) able to direct the entity to which it is attached (e.g., therapeutic agent or marker) to a target cell, for example to a specific type of tumor cell or (2) is preferentially activated at a target tissue, for example a tumor. The targeting group or targeting agent can be a small molecule, which is intended to include both non-peptides and peptides. The targeting group can also be a macromolecule, which includes saccharides, lectins, receptors, ligand for receptors, proteins such as BSA, antibodies, and so forth. In a preferred embodiment of the current invention, the targeting group is an antibody or an antibody fragment, more preferably a monoclonal antibody or monoclonal antibody fragment

The term "self-immolative spacer" refers to a bifunctional chemical moiety that is capable of covalently linking two chemical moieties into a normally stable tripartate molecule. The self-immolative spacer is capable of spontaneously separating from the second moiety if the bond to the first moiety is cleaved.

The term "detectable label" is intended to mean a moiety having a detectable physical or chemical property.

The term "cleaveable group" is intended to mean a moiety that is unstable *in vivo*. Preferably the "cleaveable group" allows for activation of the marker or therapeutic agent by cleaving the marker or agent from the rest of the conjugate. Operatively defined, the linker is preferably cleaved *in vivo* by the biological environment. The cleavage may come from any process without limitation, e.g., enzymatic, reductive, pH, etc. Preferably, the cleaveable group is selected so that activation occurs at the desired site of action, which can be a site in or near the target cells (e.g., carcinoma cells) or tissues such as at the site of therapeutic action or marker activity. Such cleavage may be enzymatic and exemplary enzymatically cleaveable groups include natural amino acids or peptide sequences that end with a natural amino acid, and are attached at their carboxyl terminus to the linker. While the degree of cleavage rate enhancement is not critical to the invention, preferred examples of cleaveable linkers are those in which at least about 10% of the cleaveable groups are cleaved in the blood stream within 24 hours of administration, most preferably at least about 35%.

The term "ligand" means any molecule that specifically binds or reactively associates or complexes with a receptor, substrate, antigenic determinant, or other binding site on a target cell or tissue. Examples of ligands include antibodies and fragments thereof (e.g., a monoclonal antibody or fragment thereof), enzymes (e.g., fibrinolytic enzymes), biologic response modifiers (e.g., interleukins, interferons, erythropeoitin, or colony stimulating factors), peptide hormones, and antigen-binding fragments thereof.

The terms "hydrazine linker" and "self-cyclizing hydrazine linker" are used interchangeably herein. These terms refer to a linker moiety that, upon a change in condition, such as a shift in pH, will undergo a cyclization reaction and form one or more rings. The hydrazine moiety is converted to a hydrazone when attached. This attachment can occur, for example, through a reaction with a ketone group on the L^4 moiety. Therefore, the term hydrazone linker can also be used to describe the linker of the current invention because of this conversion to a hydrazone upon attachment.

The term "five-membered hydrazine linker" or "5-membered hydrazine linker" refers to hydrazine-containing molecular moieties that, upon a change in condition, such as a shift in pH, will undergo a cyclization reaction and form one or more 5-membered rings.

Alternatively, this five membered linker may similarly be described as a five-membered hydrazone linker or a 5-membered hydrazone linker.

The term "six-membered hydrazine linker" or "6-membered hydrazine linker" refers to hydrazine-containing molecular moieties that, upon a change in condition such as a shift in pH, will undergo a cyclization reaction and form one or more 6-membered rings. This six membered linker may similarly be described as a six-membered hydrazone linker or a 6-membered hydrazone linker.

The term "cyclization reaction," when referring to the cyclization of a peptide, hydrazine, or disulfide linker, indicates the cyclization of that linker into a ring and initiates the separation of the drug-ligand complex. This rate can be measured *ex situ*, and is completed when at least 90%, 95%, or 100% of the product is formed.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer. These terms also encompass the term "antibody."

The term "amino acid" refers to naturally occurring and synthetic amino acids, as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, *e.g.*, hydroxyproline, γ-carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, *i.e.*, an α carbon that is bound to a hydrogen, a carboxyl group, an amino group, and an R group, *e.g.*, homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (*e.g.*, norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. One amino acid that may be used in particular is citrulline, which is a precursor to arginine and is involved in the formation of urea in the liver. Amino acid mimetics refers to chemical compounds that have a structure that is different from the general chemical structure of an amino acid, but functions in a manner similar to a naturally occurring amino acid. The term "unnatural amino acid" is intended to represent the "D" stereochemical form of the twenty naturally occurring amino acids described above. It is further understood that

the term unnatural amino acid includes homologues of the natural amino acids, and synthetically modified forms of the natural amino acids. The synthetically modified forms include, but are not limited to, amino acids having alkylene chains shortened or lengthened by up to two carbon atoms, amino acids comprising optionally substituted aryl groups, and amino acids comprised halogenated groups, preferably halogenated alkyl and aryl groups. When attached to a linker or conjugate of the invention, the amino acid is in the form of an "amino acid side chain", where the carboxylic acid group of the amino acid has been replaced with a keto (C(O)) group. Thus, for example, an alanine side chain is $-C(O)-CH(NH_2)-CH_3$, and so forth.

Amino acids and peptides may be protected by blocking groups. A blocking group is an atom or a chemical moiety that protects the N-terminus of an amino acid or a peptide from undesired reactions and can be used during the synthesis of a drug-ligand conjugate. It should remain attached to the N-terminus throughout the synthesis, and may be removed after completion of synthesis of the drug conjugate by chemical or other conditions that selectively achieve its removal. The blocking groups suitable for N-terminus protection are well known in the art of peptide chemistry. Exemplary blocking groups include, but are not limited to, hydrogen, D-amino acid, and carbobenzoxy (Cbz) chloride.

"Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (*e.g.*, degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* **19**:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* **260**: 2605-2608

(1985); Rossolini *et al.*, *Mol. Cell. Probes* **8**: 91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

The symbol ∿∿∿, whether utilized as a bond or displayed perpendicular to a bond indicates the point at which the displayed moiety is attached to the remainder of the molecule, solid support, etc.

The term "alkyl," by itself or as part of another substituent, means, unless otherwise stated, a straight or branched chain, or cyclic hydrocarbon radical, or combination thereof, which may be fully saturated, mono- or polyunsaturated and can include di- and multivalent radicals, having the number of carbon atoms designated (*i.e.* C_1-C_{10} means one to ten carbons). Examples of saturated hydrocarbon radicals include, but are not limited to, groups such as methyl, ethyl, n-propyl, isopropyl, n-butyl, t-butyl, isobutyl, sec-butyl, cyclohexyl, (cyclohexyl)methyl, cyclopropylmethyl, homologs and isomers of, for example, n-pentyl, n-hexyl, n-heptyl, n-octyl, and the like. An unsaturated alkyl group is one having one or more double bonds or triple bonds. Examples of unsaturated alkyl groups include, but are not limited to, vinyl, 2-propenyl, crotyl, 2-isopentenyl, 2-(butadienyl), 2,4-pentadienyl, 3-(1,4-pentadienyl), ethynyl, 1- and 3-propynyl, 3-butynyl, and the higher homologs and isomers. The term "alkyl," unless otherwise noted, is also meant to include those derivatives of alkyl defined in more detail below, such as "heteroalkyl." Alkyl groups, which are limited to hydrocarbon groups are termed "homoalkyl".

The term "alkylene" by itself or as part of another substituent means a divalent radical derived from an alkane, as exemplified, but not limited, by $-CH_2CH_2CH_2CH_2-$, and further includes those groups described below as "heteroalkylene." Typically, an alkyl (or alkylene) group will have from 1 to 24 carbon atoms, with those groups having 10 or fewer carbon atoms being preferred in the present invention. A "lower alkyl" or "lower alkylene" is a shorter chain alkyl or alkylene group, generally having eight or fewer carbon atoms.

The term "heteroalkyl," by itself or in combination with another term, means, unless otherwise stated, a stable straight or branched chain, or cyclic hydrocarbon radical, or combinations thereof, consisting of the stated number of carbon atoms and at least one heteroatom selected from the group consisting of O, N, Si and S, and wherein the nitrogen, carbon and sulfur atoms may optionally be oxidized and the nitrogen heteroatom may optionally be quaternized. The heteroatom(s) O, N and S and Si may be placed at any interior position of

the heteroalkyl group or at the position at which the alkyl group is attached to the remainder of the molecule. Examples include, but are not limited to, $-CH_2-CH_2-O-CH_3$, $-CH_2-CH_2-NH-CH_3$, $-CH_2-CH_2-N(CH_3)-CH_3$, $-CH_2-S-CH_2-CH_3$, $-CH_2-CH_2,-S(O)-CH_3$, $-CH_2-CH_2-S(O)_2-CH_3$, $-CH=CH-O-CH_3$, $-Si(CH_3)_3$, $-CH_2-CH=N-OCH_3$, and $-CH=CH-N(CH_3)-CH_3$. Up to two heteroatoms may be consecutive, such as, for example, $-CH_2-NH-OCH_3$ and $-CH_2-O-Si(CH_3)_3$. Similarly, the term "heteroalkylene" by itself or as part of another substituent means a divalent radical derived from heteroalkyl, as exemplified, but not limited by, $-CH_2-CH_2-S-CH_2-CH_2-$ and $-CH_2-S-CH_2-CH_2-NH-CH_2-$. For heteroalkylene groups, heteroatoms can also occupy either or both of the chain termini (*e.g.*, alkyleneoxy, alkylenedioxy, alkyleneamino, alkylenediamino, and the like). The terms "heteroalkyl" and "heteroalkylene" encompass poly(ethylene glycol) and its derivatives (see, for example, Shearwater Polymers Catalog, 2001). Still further, for alkylene and heteroalkylene linking groups, no orientation of the linking group is implied by the direction in which the formula of the linking group is written. For example, the formula $-C(O)_2R'-$ represents both $-C(O)_2R'-$ and $-R'C(O)_2-$.

The term "lower" in combination with the terms "alkyl" or "heteroalkyl" refers to a moiety having from 1 to 6 carbon atoms.

The terms "alkoxy," "alkylamino," "alkylsulfonyl," and "alkylthio" (or thioalkoxy) are used in their conventional sense, and refer to those alkyl groups attached to the remainder of the molecule via an oxygen atom, an amino group, an SO_2 group or a sulfur atom, respectively. The term "arylsulfonyl" refers to an aryl group attached to the remainder ofhte molecule via an SO_2 group, and the term "sulfhydryl" refers to an SH group.

In general, an "acyl substituent" is also selected from the group set forth above. As used herein, the term "acyl substituent" refers to groups attached to, and fulfilling the valence of a carbonyl carbon that is either directly or indirectly attached to the polycyclic nucleus of the compounds of the present invention.

The terms "cycloalkyl" and "heterocycloalkyl", by themselves or in combination with other terms, represent, unless otherwise stated, cyclic versions of substituted or unsubstituted "alkyl" and substituted or unsubstituted "heteroalkyl", respectively. Additionally, for heterocycloalkyl, a heteroatom can occupy the position at which the heterocycle is attached to the remainder of the molecule. Examples of cycloalkyl include, but are not limited to, cyclopentyl, cyclohexyl, 1-cyclohexenyl, 3-cyclohexenyl, cycloheptyl, and the like. Examples of

heterocycloalkyl include, but are not limited to, 1 –(1,2,5,6-tetrahydropyridyl), 1-piperidinyl, 2-piperidinyl, 3-piperidinyl, 4-morpholinyl, 3-morpholinyl, tetrahydrofuran-2-yl, tetrahydrofuran-3-yl, tetrahydrothien-2-yl, tetrahydrothien-3-yl, 1 –piperazinyl, 2-piperazinyl, and the like. The heteroatoms and carbon atoms of the cyclic structures are optionally oxidized.

The terms "halo" or "halogen," by themselves or as part of another substituent, mean, unless otherwise stated, a fluorine, chlorine, bromine, or iodine atom. Additionally, terms such as "haloalkyl," are meant to include monohaloalkyl and polyhaloalkyl. For example, the term "halo(C_1-C_4)alkyl" is mean to include, but not be limited to, trifluoromethyl, 2,2,2-trifluoroethyl, 4-chlorobutyl, 3-bromopropyl, and the like.

The term "aryl" means, unless otherwise stated, a substituted or unsubstituted polyunsaturated, aromatic, hydrocarbon substituent which can be a single ring or multiple rings (preferably from 1 to 3 rings) which are fused together or linked covalently. The term "heteroaryl" refers to aryl groups (or rings) that contain from one to four heteroatoms selected from N, O, and S, wherein the nitrogen, carbon and sulfur atoms are optionally oxidized, and the nitrogen atom(s) are optionally quaternized. A heteroaryl group can be attached to the remainder of the molecule through a heteroatom. Non-limiting examples of aryl and heteroaryl groups include phenyl, 1-naphthyl, 2-naphthyl, 4-biphenyl, 1-pyrrolyl, 2-pyrrolyl, 3-pyrrolyl, 3-pyrazolyl, 2-imidazolyl, 4-imidazolyl, pyrazinyl, 2-oxazolyl, 4-oxazolyl, 2-phenyl-4-oxazolyl, 5-oxazolyl, 3-isoxazolyl, 4-isoxazolyl, 5-isoxazolyl, 2-thiazolyl, 4-thiazolyl, 5-thiazolyl, 2-furyl, 3-furyl, 2-thienyl, 3-thienyl, 2-pyridyl, 3-pyridyl, 4-pyridyl, 2-pyrimidyl, 4-pyrimidyl, 5-benzothiazolyl, purinyl, 2-benzimidazolyl, 5-indolyl, 1-isoquinolyl, 5-isoquinolyl, 2-quinoxalinyl, 5-quinoxalinyl, 3-quinolyl, and 6-quinolyl. Substituents for each of the above noted aryl and heteroaryl ring systems are selected from the group of acceptable substituents described below. "Aryl" and "heteroaryl" also encompass ring systems in which one or more non-aromatic ring systems are fused, or otherwise bound, to an aryl or heteroaryl system.

For brevity, the term "aryl" when used in combination with other terms (*e.g.*, aryloxy, arylthioxy, arylalkyl) includes both aryl and heteroaryl rings as defined above. Thus, the term "arylalkyl" is meant to include those radicals in which an aryl group is attached to an alkyl group (*e.g.*, benzyl, phenethyl, pyridylmethyl and the like) including those alkyl groups in which a carbon atom (*e.g.*, a methylene group) has been replaced by, for example, an oxygen atom (*e.g.*, phenoxymethyl, 2-pyridyloxymethyl, 3-(1-naphthyloxy)propyl, and the like).

Each of the above terms (*e.g.*, "alkyl," "heteroalkyl," "aryl" and "heteroaryl") include both substituted and unsubstituted forms of the indicated radical. Preferred substituents for each type of radical are provided below.

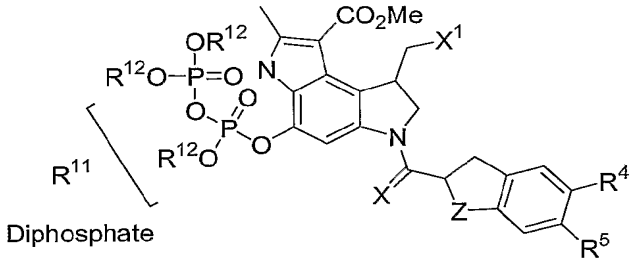
Substituents for the alkyl, and heteroalkyl radicals (including those groups often referred to as alkylene, alkenyl, heteroalkylene, heteroalkenyl, alkynyl, cycloalkyl, heterocycloalkyl, cycloalkenyl, and heterocycloalkenyl) are generally referred to as "alkyl substituents" and "heteroalkyl substituents," respectively, and they can be one or more of a variety of groups selected from, but not limited to: -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R"R"')=NR"", -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and –NO_2 in a number ranging from zero to (2m'+1), where m' is the total number of carbon atoms in such radical. R', R", R"' and R"" each preferably independently refer to hydrogen, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, e.g., aryl substituted with 1-3 halogens, substituted or unsubstituted alkyl, alkoxy or thioalkoxy groups, or arylalkyl groups. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R"' and R"" groups when more than one of these groups is present. When R' and R" are attached to the same nitrogen atom, they can be combined with the nitrogen atom to form a 5-, 6-, or 7-membered ring. For example, -NR'R" is meant to include, but not be limited to, 1-pyrrolidinyl and 4-morpholinyl. From the above discussion of substituents, one of skill in the art will understand that the term "alkyl" is meant to include groups including carbon atoms bound to groups other than hydrogen groups, such as haloalkyl (*e.g.*, -CF_3 and –CH_2CF_3) and acyl (*e.g.*, -$C(O)CH_3$, -$C(O)CF_3$, -$C(O)CH_2OCH_3$, and the like).

Similar to the substituents described for the alkyl radical, the aryl substituents and heteroaryl substituents are generally referred to as "aryl substituents" and "heteroaryl substituents," respectively and are varied and selected from, for example: halogen, -OR', =O, =NR', =N-OR', -NR'R", -SR', -halogen, -SiR'R"R"', -OC(O)R', -C(O)R', -CO_2R', -CONR'R", -OC(O)NR'R", -NR"C(O)R', -NR'-C(O)NR"R"', -NR"$C(O)_2$R', -NR-C(NR'R")=NR"', -S(O)R', -$S(O)_2$R', -$S(O)_2$NR'R", -$NRSO_2$R', -CN and –NO_2, -R', -N_3, -$CH(Ph)_2$, fluoro(C_1-C_4)alkoxy, and fluoro(C_1-C_4)alkyl, in a number ranging from zero to the total number of open valences on the aromatic ring system; and where R', R", R"' and R"" are preferably

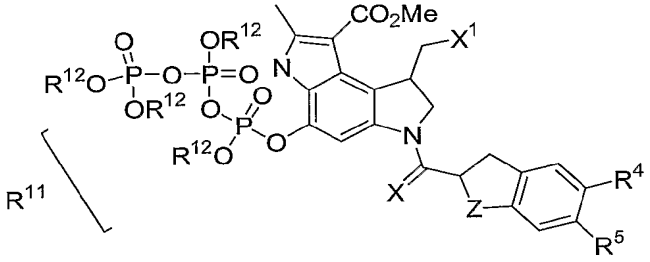
independently selected from hydrogen, (C_1-C_8)alkyl and heteroalkyl, unsubstituted aryl and heteroaryl, (unsubstituted aryl)-(C_1-C_4)alkyl, and (unsubstituted aryl)oxy-(C_1-C_4)alkyl. When a compound of the invention includes more than one R group, for example, each of the R groups is independently selected as are each R', R", R'" and R"" groups when more than one of these groups is present.

Two of the aryl substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –T-C(O)-$(CRR')_q$-U-, wherein T and U are independently –NR-, -O-, -CRR'- or a single bond, and q is an integer of from 0 to 3. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula –A-$(CH_2)_r$-B-, wherein A and B are independently –CRR'-, -O-, -NR-, -S-, -S(O)-, -$S(O)_2$-, -$S(O)_2$NR'- or a single bond, and r is an integer of from 1 to 4. One of the single bonds of the new ring so formed may optionally be replaced with a double bond. Alternatively, two of the substituents on adjacent atoms of the aryl or heteroaryl ring may optionally be replaced with a substituent of the formula -$(CRR')_s$-X-$(CR"R'")_d$-, where s and d are independently integers of from 0 to 3, and X is -O-, -NR'-, -S-, -S(O)-, -$S(O)_2$-, or –$S(O)_2$NR'-. The substituents R, R', R" and R'" are preferably independently selected from hydrogen or substituted or unsubstituted (C_1-C_6) alkyl.

As used herein, the term "diphosphate" includes but is not limited to an ester of phosphoric acid containing two phosphate groups. The term "triphosphate" includes but is not limited to an ester of phosphoric acid containing three phosphate groups. For example, particular drugs having a diphosphate or a triphosphate include:



Diphosphate



Triphosphate

As used herein, the term "heteroatom" includes oxygen (O), nitrogen (N), sulfur (S) and silicon (Si).

The symbol "R" is a general abbreviation that represents a substituent group that is selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocyclyl groups.

The term "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 a chemical agent. Pharmaceutically acceptable carriers include pharmaceutically acceptable salts, where the term "pharmaceutically acceptable salts" includes salts of the active compounds which are prepared with relatively nontoxic acids or bases, depending on the particular substituents found on the compounds described herein. When compounds of the present invention contain relatively acidic functionalities, base addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired base, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable base addition salts include sodium,

potassium, calcium, ammonium, organic amino, or magnesium salt, or a similar salt. When compounds of the present invention contain relatively basic functionalities, acid addition salts can be obtained by contacting the neutral form of such compounds with a sufficient amount of the desired acid, either neat or in a suitable inert solvent. Examples of pharmaceutically acceptable acid addition salts include those derived from inorganic acids like hydrochloric, hydrobromic, nitric, carbonic, monohydrogencarbonic, phosphoric, monohydrogenphosphoric, dihydrogenphosphoric, sulfuric, monohydrogensulfuric, hydriodic, or phosphorous acids and the like, as well as the salts derived from relatively nontoxic organic acids like acetic, propionic, isobutyric, maleic, malonic, benzoic, succinic, suberic, fumaric, lactic, mandelic, phthalic, benzenesulfonic, p-tolylsulfonic, citric, tartaric, methanesulfonic, and the like. Also included are salts of amino acids such as arginate and the like, and salts of organic acids like glucuronic or galactunoric acids and the like (*see*, for example, Berge *et al.*, "Pharmaceutical Salts", *Journal of Pharmaceutical Science*, **1977**, *66*, 1-19). Certain specific compounds of the present invention contain both basic and acidic functionalities that allow the compounds to be converted into either base or acid addition salts.

The neutral forms of the compounds are preferably regenerated by contacting the salt with a base or acid and isolating the parent compound in the conventional manner. The parent form of the compound differs from the various salt forms in certain physical properties, such as solubility in polar solvents, but otherwise the salts are equivalent to the parent form of the compound for the purposes of the present invention.

In addition to salt forms, the present invention provides compounds, which are in a prodrug form. Prodrugs of the compounds described herein are those compounds that readily undergo chemical changes under physiological conditions to provide the compounds of the present invention. Additionally, prodrugs can be converted to the compounds of the present invention by chemical or biochemical methods in an *ex vivo* environment. For example, prodrugs can be slowly converted to the compounds of the present invention when placed in a transdermal patch reservoir with a suitable enzyme or chemical reagent.

Certain compounds of the present invention can exist in unsolvated forms as well as solvated forms, including hydrated forms. In general, the solvated forms are equivalent to unsolvated forms and are encompassed within the scope of the present invention. Certain compounds of the present invention may exist in multiple crystalline or amorphous forms. In

general, all physical forms are equivalent for the uses contemplated by the present invention and are intended to be within the scope of the present invention.

Certain compounds of the present invention possess asymmetric carbon atoms (optical centers) or double bonds; the racemates, diastereomers, geometric isomers and individual isomers are encompassed within the scope of the present invention.

The compounds of the present invention may also contain unnatural proportions of atomic isotopes at one or more of the atoms that constitute such compounds. For example, the compounds may be radiolabeled with radioactive isotopes, such as for example tritium (^{3}H), iodine-125 (^{125}I) or carbon-14 (^{14}C). All isotopic variations of the compounds of the present invention, whether radioactive or not, are intended to be encompassed within the scope of the present invention.

The term "attaching moiety" or "moiety for attaching a targeting group" refers to a moiety which allows for attachment of a targeting group to the linker. Typical attaching groups include, by way of illustration and not limitation, alkyl, aminoalkyl, aminocarbonylalkyl, carboxyalkyl, hydroxyalkyl, alkyl-maleimide, alkyl-N-hydroxylsuccinimide, poly(ethylene glycol)-maleimide and poly(ethylene glycol)-N-hydroxylsuccinimide, all of which may be further substituted. The linker can also have the attaching moiety be actually appended to the targeting group.

As used herein, the term "leaving group" refers to a portion of a substrate that is cleaved from the substrate in a reaction.

The term "antibody" as referred to herein includes whole antibodies and any antigen binding fragment (*i.e.*, "antigen-binding portion") or single chains thereof. An "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (V_H) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, C_{H1}, C_{H2} and C_{H3}, and may be of the mu, delta, gamma, alpha or epsilon isotype. Each light chain is comprised of a light chain variable region (V_L) and a light chain constant region. The light chain constant region is comprised of one domain, C_L, which may be of the kappa or lambda isotype. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed

framework regions (FR). Each V_H and V_L is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (*e.g.*, effector cells) and the first component (Clq) of the classical complement system.

The terms "antibody fragment" or "antigen-binding portion" of an antibody (or simply "antibody portion"), as used herein, refers to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antibody fragment" or "antigen-binding portion" of an antibody include (i) a Fab fragment, a monovalent fragment consisting of the V_L, V_H, C_L and C_{H1} domains; (ii) a $F(ab')_2$ fragment, a bivalent fragment comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) a Fd fragment consisting of the V_H and C_{H1} domains; (iv) a Fv fragment consisting of the V_L and V_H domains of a single arm of an antibody, (v) a dAb fragment (Ward *et al.*, (1989) *Nature* 341:544-546), which consists of a V_H domain; and (vi) an isolated complementarity determining region (CDR). Furthermore, although the two domains of the Fv fragment, V_L and V_H, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the V_L and V_H regions pair to form monovalent molecules (known as single chain Fv (scFv); *see e.g.,* Bird *et al.* (1988) *Science* 242:423-426; and Huston *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:5879-5883*)*. Such single chain antibodies are also intended to be encompassed within the term "antigen-binding portion" of an antibody. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

The terms "monoclonal antibody" as used herein refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody composition displays a single binding specificity and affinity for a particular epitope.

For preparation of monoclonal or polyclonal antibodies, any technique known in the art can be used (*see, e.g.*, Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*,

Immunology Today 4: 72 (1983); Cole *et al.*, pp. 77-96 in MONOCLONAL ANTIBODIES AND CANCER THERAPY, Alan R. Liss, Inc. (1985)).

Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (*e.g.*, BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the beta subunits. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired.

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see* Kohler & Milstein, *Eur. J. Immunol.* **6**: 511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art.

In a preferred embodiment, the antibody is a chimeric or humanized antibody. Chimeric or humanized antibodies of the present invention can be prepared based on the sequence of a murine monoclonal antibody. DNA encoding the heavy and light chain immunoglobulins can be obtained from the murine hybridoma of interest and engineered to contain non-murine (*e.g.*,. human) immunoglobulin sequences using standard molecular biology techniques. For example, to create a chimeric antibody, the murine variable regions can be linked to human constant regions using methods known in the art (see *e.g.*, U.S. Patent No. 4,816,567 to Cabilly *et al.*). To create a humanized antibody, the murine CDR regions can be inserted into a human framework using methods known in the art (see *e.g.*, U.S. Patent No. 5,225,539 to Winter, and U.S. Patent Nos. 5,530,101; 5,585,089; 5,693,762 and 6,180,370 to Queen *et al.*).

In another preferred embodiment, the antibody is a human antibody. Such human antibodies can be generated by immunizing transgenic or transchromosomic mice in which the endogenous mouse immunoglobulin genes have been inactivated and exogenous human immunoglobulin genes have been introduced. Such mice are known in the art (see *e.g.*, U.S. Patent Nos. 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,877,397; 5,661,016;

5,814,318; 5,874,299; and 5,770,429; all to Lonberg and Kay; U.S. Patent Nos. 5,939,598; 6,075,181; 6,114,598; 6,150,584 and 6,162,963 to Kucherlapati *et al.*; and PCT Publication WO 02/43478 to Ishida *et al.*) Human antibodies can also be prepared using phage display methods for screening libraries of human immunoglobulin genes. Such phage display methods for isolating human antibodies also are know in the art (see *e.g.*, U.S. Patent Nos. 5,223,409; 5,403,484; and 5,571,698 to Ladner *et al.*; U.S. Patent Nos. 5,427,908 and 5,580,717 to Dower *et al.*; U.S. Patent Nos. 5,969,108 and 6,172,197 to McCafferty *et al.*; and U.S. Patent Nos. 5,885,793; 6,521,404; 6,544,731; 6,555,313; 6,582,915 and 6,593,081 to Griffiths *et al.*).

"Solid support," as used herein refers to a material that is substantially insoluble in a selected solvent system, or which can be readily separated (*e.g.*, by precipitation) from a selected solvent system in which it is soluble. Solid supports useful in practicing the present invention can include groups that are activated or capable of activation to allow selected species to be bound to the solid support. A solid support can also be a substrate, for example, a chip, wafer or well, onto which an individual, or more than one compound, of the invention is bound.

"Reactive functional group," as used herein refers to groups including, but not limited to, olefins, acetylenes, alcohols, phenols, ethers, oxides, halides, aldehydes, ketones, carboxylic acids, esters, amides, cyanates, isocyanates, thiocyanates, isothiocyanates, amines, hydrazines, hydrazones, hydrazides, diazo, diazonium, nitro, nitriles, mercaptans, sulfides, disulfides, sulfoxides, sulfones, sulfonic acids, sulfinic acids, acetals, ketals, anhydrides, sulfates, sulfenic acids isonitriles, amidines, imides, imidates, nitrones, hydroxylamines, oximes, hydroxamic acids thiohydroxamic acids, allenes, ortho esters, sulfites, enamines, ynamines, ureas, pseudoureas, semicarbazides, carbodiimides, carbamates, imines, azides, azo compounds, azoxy compounds, and nitroso compounds. Reactive functional groups also include those used to prepare bioconjugates, e.g., N-hydroxysuccinimide esters, maleimides and the like (*see*, for example, Hermanson, BIOCONJUGATE TECHNIQUES, Academic press, San Diego, 1996). Methods to prepare each of these functional groups are well known in the art and their application to or modification for a particular purpose is within the ability of one of skill in the art (*see*, for example, Sandler and Karo, eds. ORGANIC FUNCTIONAL GROUP PREPARATIONS, Academic Press, San Diego, 1989). The reactive functional groups may be protected or unprotected.

The compounds of the invention are prepared as a single isomer (*e.g.*, enantiomer, cis-trans, positional, diastereomer) or as a mixture of isomers. In a preferred embodiment, the compounds are prepared as substantially a single isomer. Methods of preparing substantially isomerically pure compounds are known in the art. For example, enantiomerically enriched mixtures and pure enantiomeric compounds can be prepared by using synthetic intermediates that are enantiomerically pure in combination with reactions that either leave the stereochemistry at a chiral center unchanged or result in its complete inversion. Alternatively, the final product or intermediates along the synthetic route can be resolved into a single stereoisomer. Techniques for inverting or leaving unchanged a particular stereocenter, and those for resolving mixtures of stereoisomers are well known in the art and it is well within the ability of one of skill in the art to choose and appropriate method for a particular situation. *See*, generally, Furniss *et al.* (eds.),VOGEL'S ENCYCLOPEDIA OF PRACTICAL ORGANIC CHEMISTRY 5TH ED., Longman Scientific and Technical Ltd., Essex, 1991, pp. 809-816; and Heller, *Acc. Chem. Res.* **23**: 128 (1990).

LINKERS

The present invention provides for drug-ligand conjugates where the drug is linked to the ligand through a chemical linker. This linker is either a peptidyl, hydrazine, or disulfide linker, and is depicted herein as $(L^4)_p$—F—$(L^1)_m$, $(L^4)_p$—H—$(L^1)_m$, or $(L^4)_p$—J—$(L^1)_m$, respectively. In addition to the linkers as being attached to the drug, the present invention also provides cleaveable linker arms that are appropriate for attachment to essentially any molecular species. The linker arm aspect of the invention is exemplified herein by reference to their attachment to a therapeutic moiety. It will, however, be readily apparent to those of skill in the art that the linkers can be attached to diverse species including, but not limited to, diagnostic agents, analytical agents, biomolecules, targeting agents, detectable labels and the like.

In one aspect, the present invention relates to linkers that are useful to attach targeting groups to therapeutic agents and markers. In another aspect, the invention provides linkers that impart stability to compounds, reduce their *in vivo* toxicity, or otherwise favorably affect their pharmacokinetics, bioavailability and/or pharmacodynamics. It is generally preferred that in such embodiments, the linker is cleaved, releasing the active drug, once the drug is delivered to its site of action. Thus, in one embodiment of the invention, the linkers of the

invention are traceless, such that once removed from the therapeutic agent or marker (such as during activation), no trace of the linker's presence remains.

In another embodiment of the invention, the linkers are characterized by their ability to be cleaved at a site in or near the target cell such as at the site of therapeutic action or marker activity. Such cleavage can be enzymatic in nature. This feature aids in reducing systemic activation of the therapeutic agent or marker, reducing toxicity and systemic side effects. Preferred cleaveable groups for enzymatic cleavage include peptide bonds, ester linkages, and disulfide linkages. In other embodiments, the linkers are sensitive to pH and are cleaved through changes in pH.

An important aspect of the current invention is the ability to control the speed with which the linkers cleave. For example, the hydrazine linkers described herein are particularly useful because, depending on which particular structure is used, one can vary the speed at which the linker cyclizes and thereby cleaves the drug from the ligand. WO 02/096910 provides several specific ligand-drug complexes having a hydrazine linker. However, there is no way to "tune" the linker composition dependent upon the rate of cyclization required, and the particular compounds described cleave the ligand from the drug at a slower rate than is preferred for many drug-linker conjugates. In contrast, the hydrazine linkers of the current invention provide for a range of cyclization rates, from very fast to very slow, thereby allowing for the selection of a particular hydrazine linker based on the desired rate of cyclization. For example, very fast cyclization can be achieved with hydrazine linkers that produce a single 5-membered ring upon cleavage. Preferred cyclization rates for targeted delivery of a cytotoxic agent to cells are achieved using hydrazine linkers that produce, upon cleavage, either two 5-membered rings or a single 6-membered ring resulting from a linker having two methyls at the geminal position. The *gem*-dimethyl effect has been shown to accelerate the rate of the cyclization reaction as compared to a single 6-membered ring without the two methyls at the geminal position. This results from the strain being relieved in the ring. Sometimes, however, substitutents may slow down the reaction instead of making it faster. Often the reasons for the retardation can be traced to steric hindrance. As shown in Example 2.4, the *gem* dimethyl substitution allows for a much faster cyclization reaction to occur compared to when the geminal carbon is a CH_2.

It is important to note, however, that in some embodiments, a linker that cleaves more slowly may be preferred. For example, in a sustained release formulation or in a

formulation with both a quick release and a slow release component, it may be useful to provide a linker which cleaves more slowly. In certain embodiments, a slow rate of cyclization is achieved using a hydrazine linker that produces, upon cleavage, either a single 6-membered ring, without the *gem*-dimethyl substitution, or a single 7-membered ring.

The linkers also serve to stabilize the therapeutic agent or marker against degradation while in circulation. This feature provides a significant benefit since such stabilization results in prolonging the circulation half-life of the attached agent or marker. The linker also serves to attenuate the activity of the attached agent or marker so that the conjugate is relatively benign while in circulation and has the desired effect, for example is toxic, after activation at the desired site of action. For therapeutic agent conjugates, this feature of the linker serves to improve the therapeutic index of the agent.

The stabilizing groups are preferably selected to limit clearance and metabolism of the therapeutic agent or marker by enzymes that may be present in blood or non-target tissue and are further selected to limit transport of the agent or marker into the cells. The stabilizing groups serve to block degradation of the agent or marker and may also act in providing other physical characteristics of the agent or marker. The stabilizing group may also improve the agent or marker's stability during storage in either a formulated or non-formulated form.

Ideally, the stabilizing group is useful to stabilize a therapeutic agent or marker if it serves to protect the agent or marker from degradation when tested by storage of the agent or marker in human blood at 37°C for 2 hours and results in less than 20%, preferably less than 10%, more preferably less than 5% and even more preferably less than 2%, cleavage of the agent or marker by the enzymes present in the human blood under the given assay conditions.

The present invention also relates to conjugates containing these linkers. More particularly, the invention relates to prodrugs that may be used for the treatment of disease, especially for cancer chemotherapy. Specifically, use of the linkers described herein provide for prodrugs that display a high specificity of action, a reduced toxicity, and an improved stability in blood relative to prodrugs of similar structure.

The linkers of the present invention as described herein may be present at any position within the cytotoxic conjugate.

Thus, there is provided a linker that may contain any of a variety of groups as part of its chain that will cleave *in vivo*, e.g., in the blood stream at a rate which is enhanced relative

to that of constructs that lack such groups. Also provided are conjugates of the linker arms with therapeutic and diagnostic agents. The linkers are useful to form prodrug analogs of therapeutic agents and to reversibly link a therapeutic or diagnostic agent to a targeting agent, a detectable label, or a solid support. The linkers may be incorporated into complexes that include the cytotoxins of the invention.

In addition to the cleaveable peptide, hydrazine, or disulfide group, one or more self-immolative linker groups L^1 are optionally introduced between the cytotoxin and the targeting agent. These linker groups may also be described as spacer groups and contain at least two reactive functional groups. Typically, one chemical functionality of the spacer group bonds to a chemical functionality of the therapeutic agent, *e.g.*, cytotoxin, while the other chemical functionality of the spacer group is used to bond to a chemical functionality of the targeting agent or the cleaveable linker. Examples of chemical functionalities of spacer groups include hydroxy, mercapto, carbonyl, carboxy, amino, ketone, and mercapto groups.

The self-immolative linkers, represented by L^1, are generally substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl or a substituted or unsubstituted heteroalkyl group. In one embodiment, the alkyl or aryl groups may comprise between 1 and 20 carbon atoms. They may also comprise a polyethylene glycol moiety.

Exemplary spacer groups include, for example, 6-aminohexanol, 6-mercaptohexanol, 10-hydroxydecanoic acid, glycine and other amino acids, 1,6-hexanediol, β-alanine, 2-aminoethanol, cysteamine (2-aminoethanethiol), 5-aminopentanoic acid, 6-aminohexanoic acid, 3-maleimidobenzoic acid, phthalide, α-substituted phthalides, the carbonyl group, aminal esters, nucleic acids, peptides and the like.

The spacer can serve to introduce additional molecular mass and chemical functionality into the cytotoxin-targeting agent complex. Generally, the additional mass and functionality will affect the serum half-life and other properties of the complex. Thus, through careful selection of spacer groups, cytotoxin complexes with a range of serum half-lives can be produced.

The spacer(s) located directly adjacent to the drug moiety is also denoted as $(L^1)_m$, wherein m is an integer selected from 0, 1, 2, 3, 4, 5, or 6. When multiple L^1 spacers are present, either identical or different spacers may be used. L^1 may be any self-immolative group.

In one embodiment, L^1 is preferably is a substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl, unsubstituted heterocycloalkyl, and substituted heterocycloalkyl. When the drug-ligand conjugate comprises a hydrazine linker, L^1 does not comprise a disulfide bond.

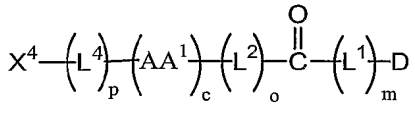
L^4 is a linker moiety that imparts increased solubility or decreased aggregation properties to conjugates utilizing a linker that contains the moiety. The L^4 linker does not have to be self immolative. In one embodiment, the L^4 moiety is substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroalkyl, or unsubstituted heteroalkyl, any of which may be straight, branched, or cyclic. The substitutions may be, for example, a lower (C^1-C^6) alkyl, alkoxy, aklylthio, alkylamino, or dialkylamino. In certain embodiments, L^4 comprises a non-cyclic moiety . In another embodiment, L^4 comprises any positively or negatively charged amino acid polymer, such as polylysine or polyargenine. L^4 can comprise a polymer such as a polyethylene glycol moiety. Additionally the L^4 linker comprises, for example, both a polymer component and a small chemical moiety.

In a preferred embodiment, L^4 comprises a polyethylene glycol (PEG) moiety. The PEG portion of L^4 may be between 1 and 50 units long. Preferably, the PEG will have 1-12 repeat units, more preferably 3-12 repeat units, more preferably 2-6 repeat units, or even more preferably 3-5 repeat units and most preferably 4 repeat units. L^4 may consist solely of the PEG moiety, or it may also contain an additional substituted or unsubstituted alkyl or heteroalkyl. It is useful to combine PEG as part of the L^4 moiety to enhance the water solubility of the complex. Additionally, the PEG moiety reduces the degree of aggregation that may occur during the conjugation of the drug to the antibody.

(1) Peptide Linkers (F)

As discussed above, the peptidyl linkers of the invention can be represented by the general formula: $(L^4)_p$—F— $(L^1)_m$, wherein F represents the linker portion comprising the peptidyl moiety. In one embodiment, the F portion comprises an optional additional self-immolative linker(s), L^2, and a carbonyl group. In another embodiment, the F portion comprises an amino group and an optional spacer group(s), L^3.

Accordingly, in one embodiment, the conjugate comprising the peptidyl linker comprises a structure of the Formula 4:

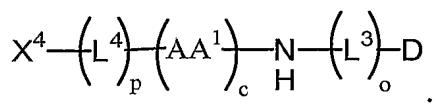


(4)

In this embodiment, $L^1$is a self-immolative linker, as described above, and L^4 is a moiety that imparts increased solubility, or decreased aggregation properties, as described above. L^2 represents a self-immolative linker(s). m is 0, 1, 2, 3, 4, 5, or 6; o and p are independently 0 or 1. In one embodiment, m is 3, 4, 5 or 6. AA^1 represents one or more natural amino acids, and/or unnatural α-amino acids; c is an integer between 1 and 20.

In the peptide linkers of the invention of the above Formula 4, AA^1 is linked, at its amino terminus, either directly to L^4 or, when L^4 is absent, directly to the X^4 group (*i.e.*, the targeting agent, detectable label, protected reactive functional group or unprotected reactive functional group). In some embodiments, when L^4 is present, L^4 does not comprise a carboxylic acyl group directly attached to the N-terminus of $(AA^1)_c$. Thus, it is not necessary in these embodiments for there to be a carboxylic acyl unit directly between either L^4 or X^4 and AA^1, as is necessary in the peptidic linkers of U.S. Patent No. 6,214,345.

In another embodiment, the conjugate comprising the peptidyl linker comprises a structure of the Formula 5:



(5)

In this embodiment, L^4 is a moiety that imparts increased solubility, or decreased aggregation properties, as described above; L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group, and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a

pendant amine functional group of D; and o and p are independently 0 or 1. AA^1 represents one or more natural amino acids, and/or unnatural α-amino acids; c is an integer between 1 and 20. In this embodiment, L^1 is absent (*i.e.*, m is 0 is the general formula).

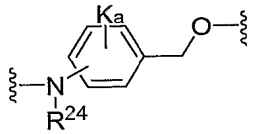
In the peptide linkers of the invention of the above Formula 5, AA^1 is linked, at its amino terminus, either directly to L^4 or, when L^4 is absent, directly to the X^4 group (*i.e.*, the targeting agent, detectable label, protected reactive functional group or unprotected reactive functional group). In some embodiments, when L^4 is present, L^4 does not comprise a carboxylic acyl group directly attached to the N-terminus of $(AA^1)_c$. Thus, it is not necessary in these embodiments for there to be a carboxylic acyl unit directly between either L^4 or X^4 and AA^1, as is necessary in the peptidic linkers of U.S. Patent No. 6,214,345.

The self-immolative linker L^2

The self-immolative linker L^2 is a bifunctional chemical moiety which is capable of covalently linking together two spaced chemical moieties into a normally stable tripartate molecule, releasing one of said spaced chemical moieties from the tripartate molecule by means of enzymatic cleavage; and following said enzymatic cleavage, spontaneously cleaving from the remainder of the molecule to release the other of said spaced chemical moieties. In accordance with the present invention, the self-immolative spacer is covalently linked at one of its ends to the peptide moiety and covalently linked at its other end to the chemical reactive site of the drug moiety whose derivatization inhibits pharmacological activity, so as to space and covalently link together the peptide moiety and the drug moiety into a tripartate molecule which is stable and pharmacologically inactive in the absence of the target enzyme, but which is enzymatically cleavable by such target enzyme at the bond covalently linking the spacer moiety and the peptide moiety to thereby effect release of the peptide moiety from the tripartate molecule. Such enzymatic cleavage, in turn, will activate the self-immolating character of the spacer moiety and initiate spontaneous cleavage of the bond covalently linking the spacer moiety to the drug moiety, to thereby effect release of the drug in pharmacologically active form.

The self-immolative linker L^2 may be any self-immolative group. Preferably L^2 is a substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, unsubstituted heterocycloalkyl, substituted heterocycloalkyl, substituted and unsubstituted aryl, and substituted and unsubstituted heteroaryl.

One particularly preferred self-immolative spacer L^2 may be represented by the formula 6:

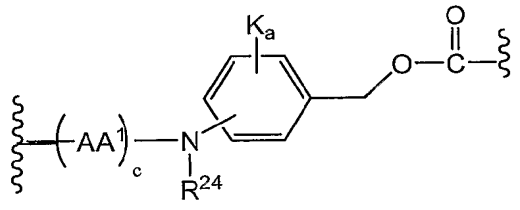


(6)

The aromatic ring of the aminobenzyl group may be substituted with one or more "K" groups. A "K" group is a substituent on the aromatic ring that replaces a hydrogen otherwise attached to one of the four non-substituted carbons that are part of the ring structure. The "K" group may be a single atom, such as a halogen, or may be a multi-atom group, such as alkyl, heteroalkyl, amino, nitro, hydroxy, alkoxy, haloalkyl, and cyano. Each K is independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}, wherein R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl and unsubstituted heterocycloalkyl. Exemplary K substituents include, but are not limited to, F, Cl, Br, I, NO_2, OH, OCH_3, $NHCOCH_3$, $N(CH_3)_2$, $NHCOCF_3$ and methyl. For "Ka", a is an integer of 0, 1, 2, 3, or 4. In one preferred embodiment, a is 0.

The ether oxygen atom of the structure shown above is connected to a carbonyl group. The line from the NR^{24} functionality into the aromatic ring indicates that the amine functionality may be bonded to any of the five carbons that both form the ring and are not substituted by the $-CH_2-O-$ group. Preferably, the NR^{24} functionality of X is covalently bound to the aromatic ring at the para position relative to the $-CH_2-O-$ group. R^{24} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. In a specific embodiment, R^{24} is hydrogen.

In a preferred embodiment, the invention provides a peptide linker of formula (4) above, wherein F comprises the structure:



wherein

R^{24} is selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl;

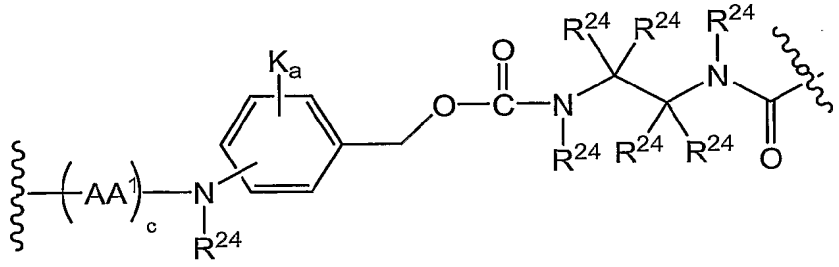
Each K is a member independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}

wherein

R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl; and

a is an integer of 0,1, 2, 3, or 4.

In another embodiment, the peptide linker of formula (4) above comprises a $-F-(L^1)_m-$ that comprises the structure:

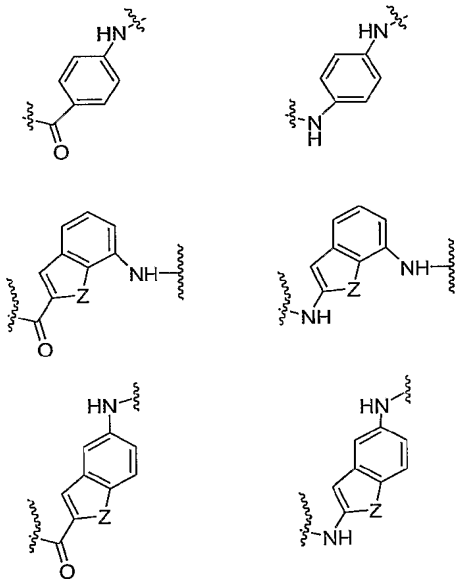


wherein

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl.

The Spacer Group L^3

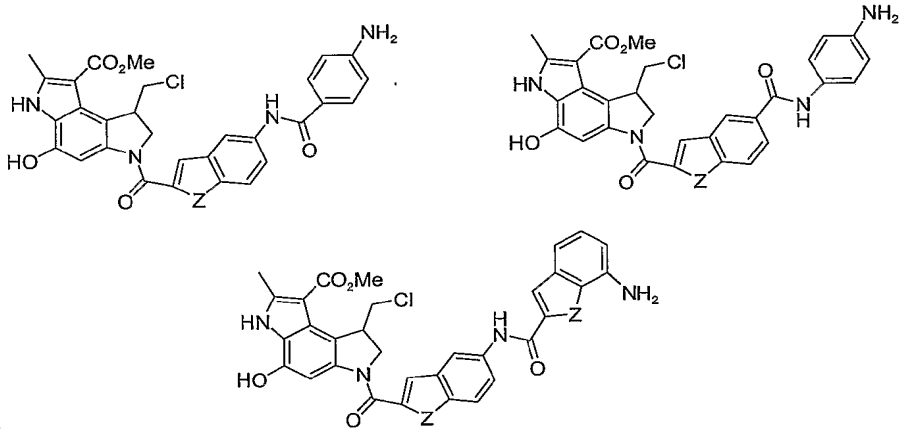
The spacer group L^3 is characterized in that it comprises a primary or secondary amine or a carboxyl functional group, and either the amine of the L^3 group forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D. L^3 can be selected from the group consisting of substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted hteroaryl, or substituted or unsubstituted heterocycloalkyl. In a preferred embodiment, L^3 comprises an aromatic group. More preferably, L^3 comprises a benzoic acid group, an aniline group or indole group. Non-limiting examples of structures that can serve as an -L^3-NH- spacer include the following structures:



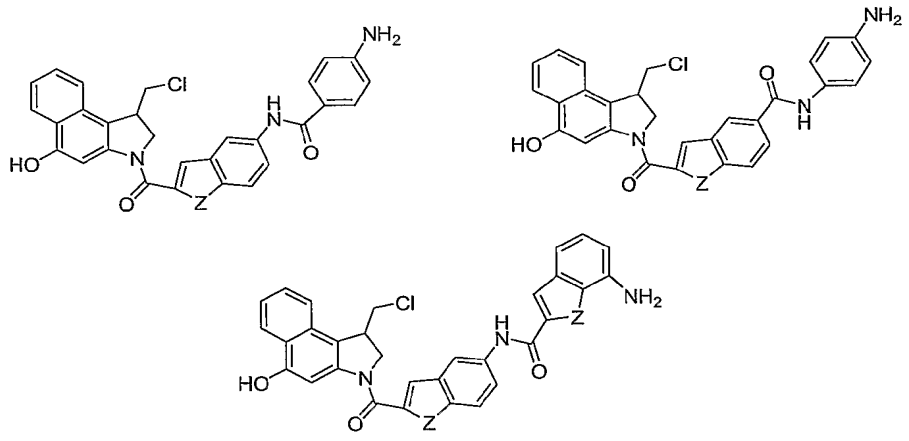
wherein Z is a member selected from O, S and NR^{23}, and

wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

Upon cleavage of the linker of the invention containing L^3, the L^3 moiety remains attached to the drug, D. Accordingly, the L^3 moiety is chosen such that its presence attached to D does not significantly alter the activity of D. In another embodiment, a portion of the drug D itself functions as the L^3 spacer. For example, in one embodiment, the drug, D, is a duocarmycin derivative in which a portion of the drug functions as the L^3 spacer. Non-limiting examples of such embodiments include those in which NH_2-(L^3)-D has a structure selected from the group consisting of:



and



wherein Z is a member selected from O, S and NR^{23},

wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and

wherein the NH_2 group on each structure reacts with $(AA^1)_c$ to form $-(AA^1)_c$-NH-.

The Peptide Sequence AA^1

The group AA^1 represents a single amino acid or a plurality of amino acids that are joined together by amide bonds. The amino acids may be natural amino acids and/or unnatural α-amino acids.

The peptide sequence $(AA^1)_c$ is functionally the amidification residue of a single amino acid (when c=1) or a plurality of amino acids joined together by amide bonds. The peptide of the current invention is selected for directing enzyme-catalyzed cleavage of the peptide by an enzyme in a location of interest in a biological system. For example, for conjugates that are targeted to a cell using a targeting agent, and then taken up by the cell, a peptide is chosen that is cleaved by one or more lysosomal proteases such that the peptide is cleaved intracellularly within the lysosome. The number of amino acids within the peptide can range from 1 to 20; but more preferably there will be 2-8 amino acids, 2-6 amino acids or 2, 3 or 4 amino acids comprising $(AA^1)_c$. Peptide sequences that are susceptible to cleavage by specific enzymes or classes of enzymes are well known in the art.

Many peptide sequences that are cleaved by enzymes in the serum, liver, gut, etc. are known in the art. An exemplary peptide sequence of the invention includes a peptide sequence that is cleaved by a protease. The focus of the discussion that follows on the use of a protease-sensitive sequence is for clarity of illustration and does not serve to limit the scope of the present invention.

When the enzyme that cleaves the peptide is a protease, the linker generally includes a peptide containing a cleavage recognition sequence for the protease. A cleavage recognition sequence for a protease is a specific amino acid sequence recognized by the protease during proteolytic cleavage. Many protease cleavage sites are known in the art, and these and other cleavage sites can be included in the linker moiety. *See, e.g.*, Matayoshi *et al. Science* **247**: 954 (1990); Dunn *et al. Meth. Enzymol.* **241**: 254 (1994); Seidah *et al. Meth. Enzymol.* **244**: 175

(1994); Thornberry, *Meth. Enzymol.* **244**: 615 (1994); Weber *et al. Meth. Enzymol.* **244**: 595 (1994); Smith *et al. Meth. Enzymol.* **244**: 412 (1994); Bouvier *et al. Meth. Enzymol.* **248**: 614 (1995), Hardy *et al.*, in AMYLOID PROTEIN PRECURSOR IN DEVELOPMENT, AGING, AND ALZHEIMER'S DISEASE, ed. Masters *et al.* pp. 190-198 (1994).

The amino acids of the peptide sequence $(AA^1)_c$ are chosen based on their suitability for selective enzymatic cleavage by particular molecules such as tumor-associated protease. The amino acids used may be natural or unnatural amino acids. They may be in the L or the D configuration. In one embodiment, at least three different amino acids are used. In another embodiment, only two amino acids are used.

In a preferred embodiment, the peptide sequence $(AA^1)_c$ is chosen based on its ability to be cleaved by a lysosomal proteases, non-limiting examples of which include cathepsins B, C, D, H, L and S. Preferably, the peptide sequence $(AA^1)_c$ is capable of being cleaved by cathepsin B *in vitro*, which can be tested using *in vitro* protease cleavage assays known in the art.

In another embodiment, the peptide sequence $(AA^1)_c$ is chosen based on its ability to be cleaved by a tumor-associated protease, such as a protease that is found extracellularly in the vicinity of tumor cells, non-limiting examples of which include thimet oligopeptidase (TOP) and CD10. The ability of a peptide to be cleaved by TOP or CD10 can be tested using *in vitro* protease cleavage assays known in the art.

Suitable, but non-limiting, examples of peptide sequences suitable for use in the conjugates of the invention include Val-Cit, Val-Lys, Phe-Lys, Lys-Lys, Ala-Lys, Phe-Cit, Leu-Cit, Ile-Cit, Trp, Cit, Phe-Ala, Phe-N^9-tosyl-Arg, Phe-N^9-nitro-Arg, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Leu-Ala-Leu, Ile-Ala-Leu, Val-Ala-Val, Ala-Leu-Ala-Leu (SEQ ID NO: 1), β-Ala-Leu-Ala-Leu (SEQ ID NO: 2) and Gly-Phe-Leu-Gly (SEQ ID NO: 3). Preferred peptides sequences are Val-Cit and Val-Lys.

In another embodiment, the amino acid located the closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val. In yet another embodiment, the amino acid located the closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cys, Gln, Glu, Gly, Ile, Leu, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

Proteases have been implicated in cancer metastasis. Increased synthesis of the protease urokinase was correlated with an increased ability to metastasize in many cancers. Urokinase activates plasmin from plasminogen, which is ubiquitously located in the extracellular space and its activation can cause the degradation of the proteins in the extracellular matrix through which the metastasizing tumor cells invade. Plasmin can also activate the collagenases thus promoting the degradation of the collagen in the basement membrane surrounding the capillaries and lymph system thereby allowing tumor cells to invade into the target tissues (Dano, *et al. Adv. Cancer. Res.*, **44**: 139 (1985)). Thus, it is within the scope of the present invention to utilize as a linker a peptide sequence that is cleaved by urokinase.

The invention also provides the use of peptide sequences that are sensitive to cleavage by tryptases. Human mast cells express at least four distinct tryptases, designated α βI, βII, and βIII. These enzymes are not controlled by blood plasma proteinase inhibitors and only cleave a few physiological substrates *in vitro*. The tryptase family of serine proteases has been implicated in a variety of allergic and inflammatory diseases involving mast cells because of elevated tryptase levels found in biological fluids from patients with these disorders. However, the exact role of tryptase in the pathophysiology of disease remains to be delineated. The scope of biological functions and corresponding physiological consequences of tryptase are substantially defined by their substrate specificity.

Tryptase is a potent activator of pro-urokinase plasminogen activator (uPA), the zymogen form of a protease associated with tumor metastasis and invasion. Activation of the plasminogen cascade, resulting in the destruction of extracellular matrix for cellular extravasation and migration, may be a function of tryptase activation of pro-urokinase plasminogen activator at the P4-P1 sequence of Pro-Arg-Phe-Lys (SEQ ID NO: 4) (Stack, *et al.*, *Journal of Biological Chemistry* **269** (13): 9416-9419 (1994)). Vasoactive intestinal peptide, a neuropeptide that is implicated in the regulation of vascular permeability, is also cleaved by tryptase, primarily at the Thr-Arg-Leu-Arg (SEQ ID NO: 5) sequence (Tam, *et al.*, *Am. J. Respir. Cell Mol. Biol.* **3**: 27-32 (1990)). The G-protein coupled receptor PAR-2 can be cleaved and activated by tryptase at the Ser-Lys-Gly-Arg (SEQ ID NO: 6) sequence to drive fibroblast proliferation, whereas the thrombin activated receptor PAR-1 is inactivated by tryptase at the Pro-Asn-Asp-Lys (SEQ ID NO: 7) sequence (Molino *et al.*, *Journal of Biological Chemistry*

272(7): 4043-4049 (1997)). Taken together, this evidence suggests a central role for tryptase in tissue remodeling as a consequence of disease. This is consistent with the profound changes observed in several mast cell-mediated disorders. One hallmark of chronic asthma and other long-term respiratory diseases is fibrosis and thickening of the underlying tissues that could be the result of tryptase activation of its physiological targets. Similarly, a series of reports have shown angiogenesis to be associated with mast cell density, tryptase activity and poor prognosis in a variety of cancers (Coussens *et al.*, *Genes and Development* **13**(11): 1382-97 (1999)); Takanami *et al.*, *Cancer* **88**(12): 2686-92 (2000); Toth-Jakatics *et al.*, *Human Pathology* **31**(8): 955-960 (2000); Ribatti *et al.*, *International Journal of Cancer* **85**(2): 171-5 (2000)).

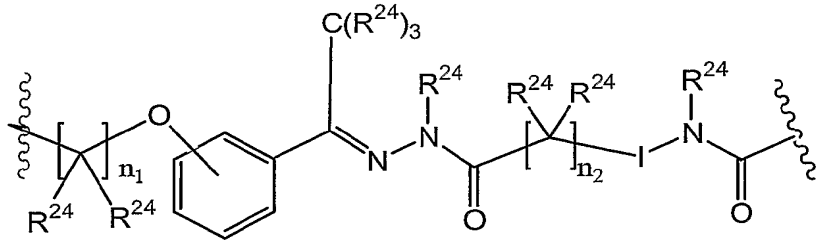
Methods are known in the art for evaluating whether a particular protease cleaves a selected peptide sequence. For example, the use of 7-amino-4-methyl coumarin (AMC) fluorogenic peptide substrates is a well-established method for the determination of protease specificity (Zimmerman, M., *et al.*, (1977) *Analytical Biochemistry* **78**:47-51). Specific cleavage of the anilide bond liberates the fluorogenic AMC leaving group allowing for the simple determination of cleavage rates for individual substrates. More recently, arrays (Lee, D., *et al.*, (1999) *Bioorganic and Medicinal Chemistry Letters* **9**:1667-72) and positional-scanning libraries (Rano, T.A., *et al.*, (1997) *Chemistry and Biology* **4**:149-55) of AMC peptide substrate libraries have been employed to rapidly profile the N-terminal specificity of proteases by sampling a wide range of substrates in a single experiment. Thus, one of skill in the art may readily evaluate an array of peptide sequences to determine their utility in the present invention without resort to undue experimentation.

(2) Hydrazine Linkers (H)

In a second embodiment, the conjugate of the invention comprises a hydrazine self-immolative linker, wherein the conjugate has the structure:

$$X^4\text{——}(L^4)_p\text{—H—}(L^1)_m\text{——D}$$

wherein D, L^1, L^4, and X^4 are as defined above and described further herein, and H is a linker comprising the structure:



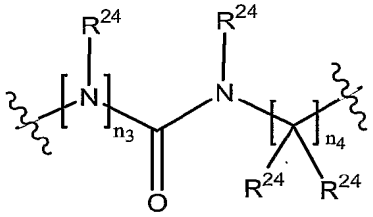
wherein

n_1 is an integer from 1 – 10;

n_2 is 0, 1, or 2;

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl; and

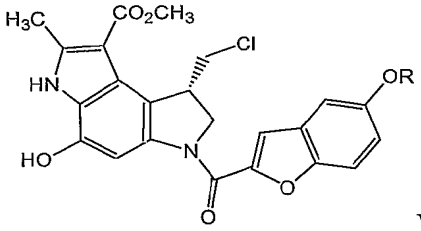
I is either a bond (*i.e.*, the bond between the carbon of the backbone and the adjacent nitrogen) or:



wherein n_3 is 0 or 1, with the proviso that when n_3 is 0, n_2 is not 0; and

n_4 is 1, 2, or 3,

wherein when I is a bond, n_1 is 3 and n_2 is 1, D can not be



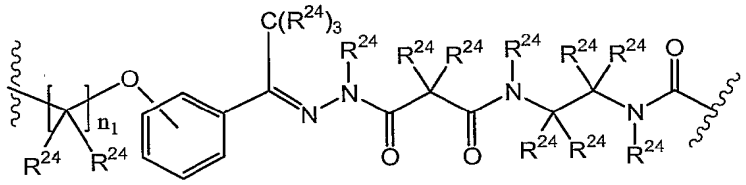
where R is Me or CH_2- CH_2-NMe_2.

In one embodiment, the substitution on the phenyl ring is a para substitution. In preferred embodiments, n_1 is 2, 3, or 4 or n_1 is 3. In preferred embodiments, n_2 is 1. In preferred

embodiments, I is a bond (*i.e.*, the bond between the carbon of the backbone and the adjacent nitrogen). In one aspect, the hydrazine linker, H, can form a 6-membered self immolative linker upon cleavage, for example, when n_3 is 0 and n_4 is 2. In another aspect, the hydrazine linker, H, can form two 5-membered self immolative linkers upon cleavage. In yet other aspects, H forms a 5-membered self immolative linker, H forms a 7-membered self immolative linker, or H forms a 5-membered self immolative linker and a 6-membered self immolative linker, upon cleavage. The rate of cleavage is affected by the size of the ring formed upon cleavage. Thus, depending upon the rate of cleavage desired, an appopriate size ring to be formed upon cleavage can be selected.

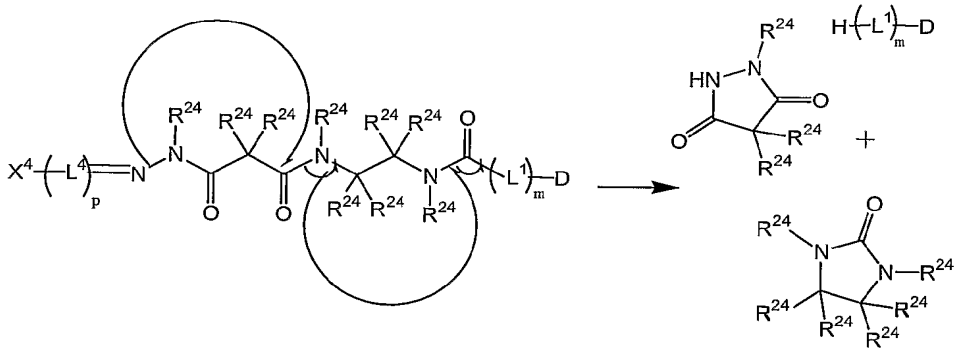
Five Membered Hydrazine Linkers

In one embodiment, the hydrazine linker comprises a 5-membered hydrazine linker, wherein H comprises the structure:

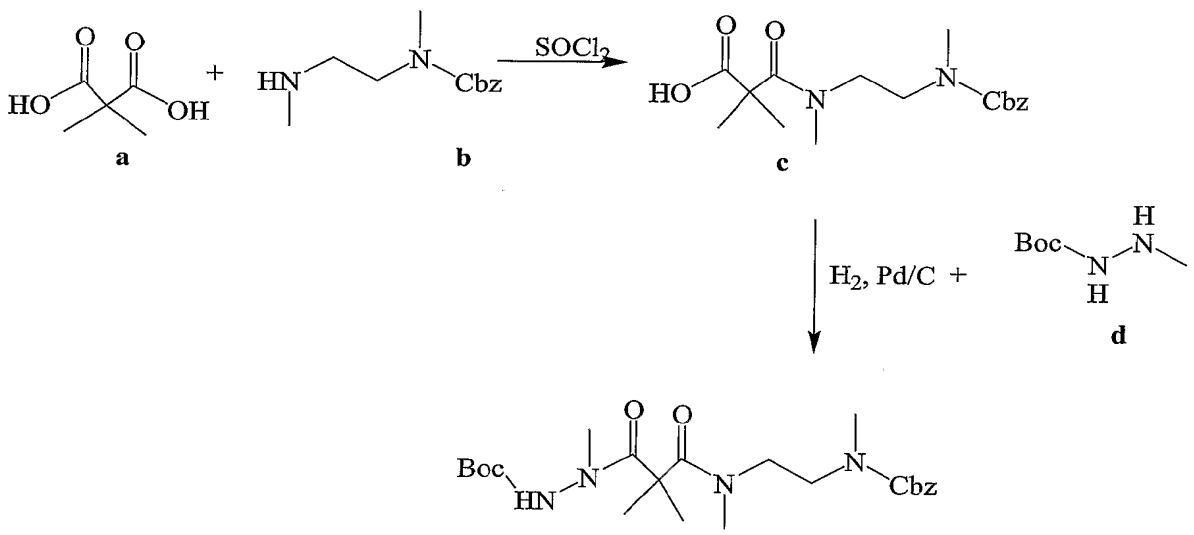


In a preferred embodiment, n_1 is 2, 3, or 4. In another preferred embodiment, n_1 is 3. In the above structure, each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. In one embodiment, each R^{24} is independently H or a $C_1 - C_6$ alkyl. In another embodiment, each R^{24} is independently H or a $C_1 - C_3$ alkyl, more preferably H or CH_3. In another embodiment, at least one R^{24} is a methyl group. In another embodiment, each R_{24} is H. Each R^{24} is selected to tailor the compounds steric effects and for altering solubility.

The 5-membered hydrazine linkers can undergo one or more cyclization reactions that separate the drug from the linker, and can be described, for example, by:



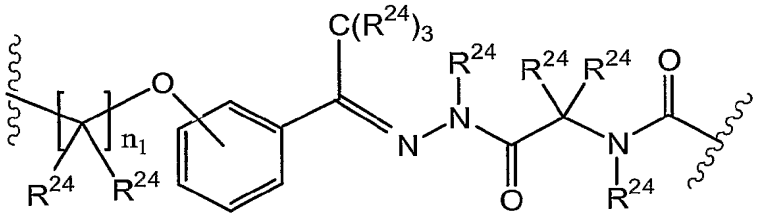
An exemplary synthetic route for preparing a five membered linker of the invention is:



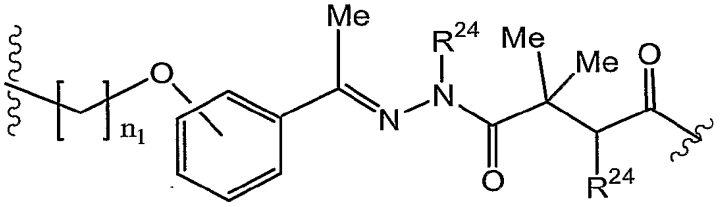
The Cbz-protected DMDA **b** is reacted with 2,2-Dimethyl-malonic acid **a** in solution with thionyl chloride to form a Cbz-DMDA-2,2-dimethylmalonic acid **c**. Compound **c** is reacted with Boc-N-methyl hydrazine **d** in the presence of hydrogen to form DMDA-2,2-dimetylmalonic-Boc-N-methylhydrazine **e**.

Six Membered Hydrazine Linkers

In another embodiment, the hydrazine linker comprises a 6-membered hydrazine linker, wherein H comprises the structure:

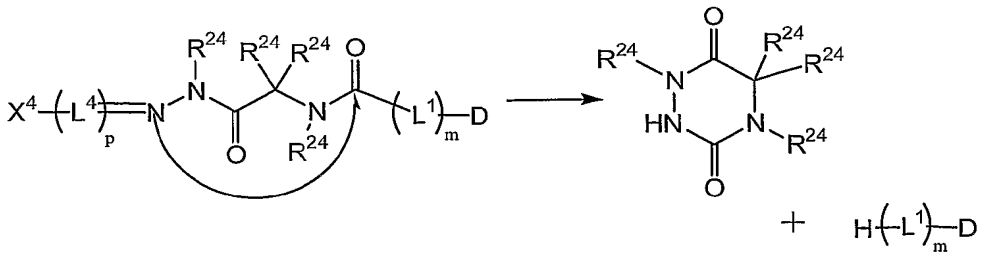


In a preferred embodiment, n_1 is 3. In the above structure, each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. In one embodiment, each R^{24} is independently H or a $C_1 - C_6$ alkyl. In another embodiment, each R^{24} is independently H or a $C_1 - C_3$ alkyl, more preferably H or CH_3. In another embodiment, at least one R^{24} is a methyl group. In another embodiment, each R_{24} is H. Each R^{24} is selected to tailor the compounds steric effects and for altering solubility. In a preferred embodiment, H comprises the structure:

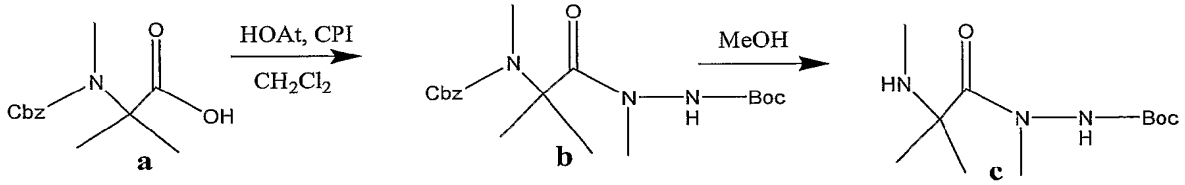


In one embodiment, H comprises a geminal dimethyl substitution. In one embodiment of the above structure, each R^{24} independently an H or a substituted or unsubstituted alkyl.

The 6-membered hydrazine linkers will undergo a cyclization reaction that separates the drug from the linker, and can be described as:



An exemplary synthetic route for preparing a six membered linker of the invention is:

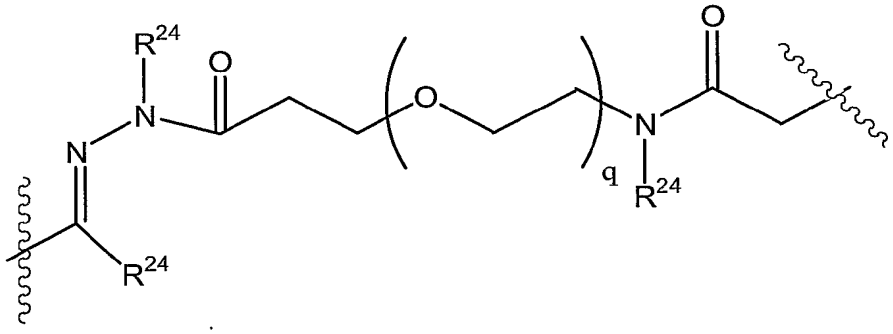


The Cbz-protected dimethyl alanine **a** in solution with dichlormethane, was reacted with HOAt, and CPI to form a Cbz-protected dimethylalanine hydrazine **b**. The hydrazine **b** is deprotected by the action of methanol, forming compound **c**.

Other Hydrazine Linkers

It is contemplated that the invention comprises a linker having seven members. This linker would likely not cyclize as quickly as the five or six membered linkers, but this may be preferred for some drug-ligand conjugates. Similarly, the hydrazine linker may comprise two six membered rings or a hydrazine linker having one six and one five membered cyclization products. A five and seven membered linker as well as a six and seven membered linker are also contemplated.

Another hydrazine structure, H, has the formula:



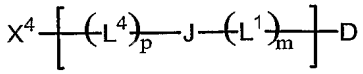
where q is 0, 1,2, 3, 4, 5, or 6; and

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl.

This hydrazine structure can also form five-, six-, or seven-membered rings and additional components can be added to form multiple rings.

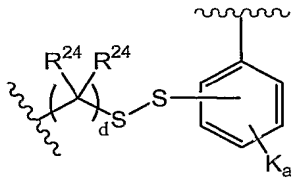
(3) Disulfide Linkers (J)

In yet another embodiment, the linker comprises an enzymatically cleavable disulfide group. In one embodiment, the invention provides a cytotoxic drug-ligand compound having a structure according to Formula 3:



(3)

wherein D, L^1, L^4, and X^4 are as defined above and described further herein, and J is a disulfide linker comprising a group having the structure:



wherein

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl;

each K is a member independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}

wherein

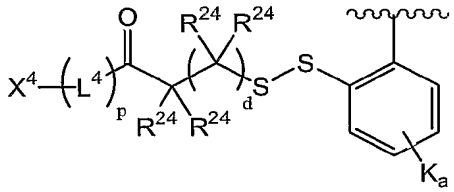
R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl and unsubstituted heterocycloalkyl;

a is an integer of 0,1, 2, 3, or 4; and

d is an integer of 0, 1, 2, 3, 4, 5, or 6.

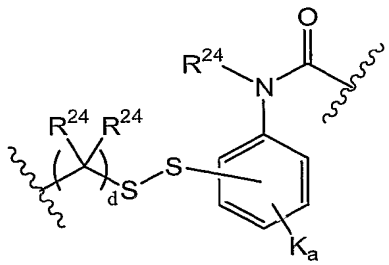
The aromatic ring of the disulfides linker may be substituted with one or more "K" groups. A "K" group is a substituent on the aromatic ring that replaces a hydrogen otherwise attached to one of the four non-substituted carbons that are part of the ring structure. The "K" group may be a single atom, such as a halogen, or may be a multi-atom group, such as alkyl, heteroalkyl, amino, nitro, hydroxy, alkoxy, haloalkyl, and cyano. Exemplary K substituents independently include, but are not limited to, F, Cl, Br, I, NO_2, OH, OCH_3, $NHCOCH_3$, $N(CH_3)_2$, $NHCOCF_3$ and methyl. For "Ka", a is an integer of 0, 1, 2, 3, or 4. In a specific embodiment, a is 0.

In a preferred embodiment, the linker comprises an enzymatically cleavable disulfide group of the following formula:

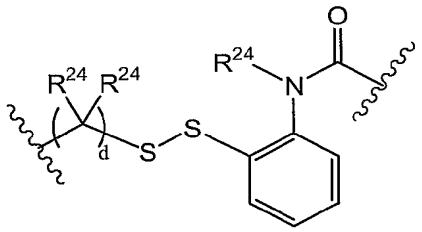


In this embodiment, the identities of L^4, X^4, p, and R^{24} are as described above, and d is 0, 1, 2, 3, 4, 5, or 6. In a particular embodiment, d is 1 or 2.

A more specific disulfide linker is shown in the formula below:

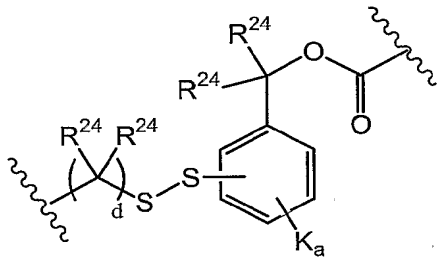


A specific example of this embodiment is as follows:

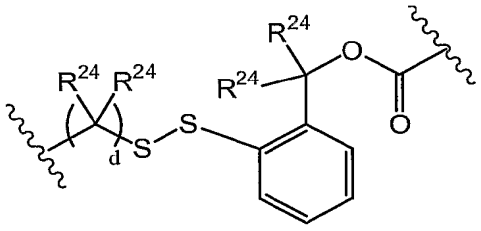


Preferably, d is 1 or 2.

Another disulfide linker is shown in the formula below:



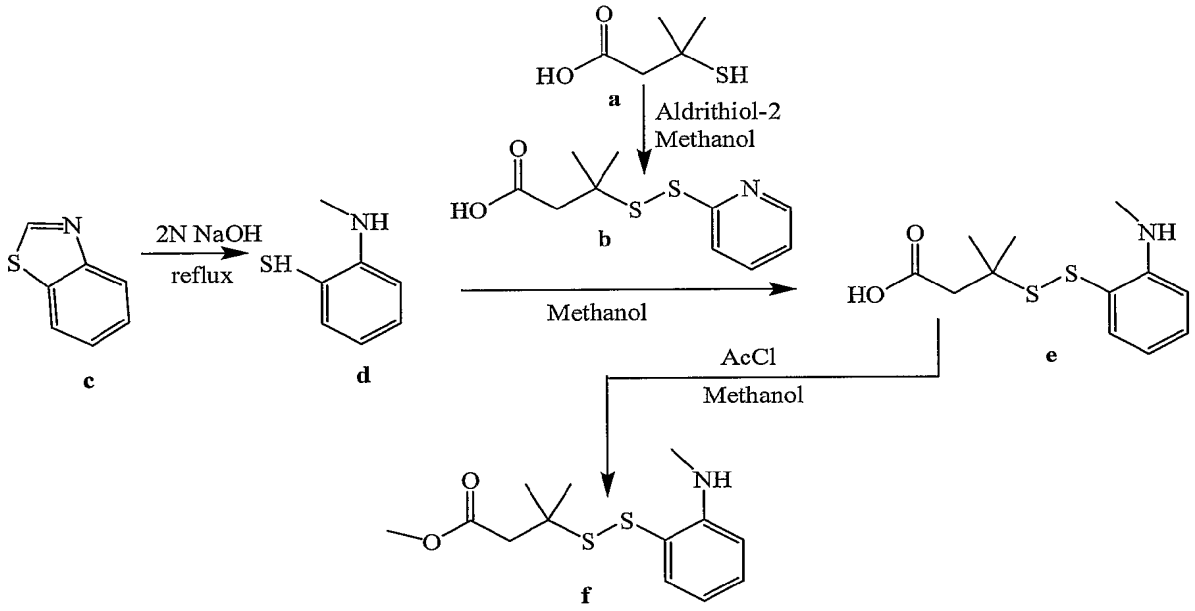
A specific example of this embodiment is as follows:



Preferably, d is 1 or 2.

In various embodiments, the disulfides are ortho to the amine. In another specific embodiment, a is 0. In preferred embodiments, R^{24} is independently selected from H and CH_3.

An exemplary synthetic route for preparing a disulfide linker of the invention is as follows:



A solution of 3-mercaptopropionic acid **a** is reacted with aldrithiol-2 to form 3-methyl benzothiazolium iodide **b**. 3-methylbenzothiazolium iodide **c** is reacted with sodium hydroxide to form compound **d**. A solution of compound **d** with methanol is further reacted with compound **b** to form compound **e**. Compound **e** deprotected by the action of acetyl chloride and methanol forming compound **f**.

The drug-ligand conjugate of the current invention may optionally contain two or more linkers. These linkers may be the same or different. For example, a peptidyl linker may be used to connect the drug to the ligand and a second peptidyl linker may attach a diagnostic agent the complex. Alternatively, any of a peptidyl, hydrazine, or disulfide linker may connect the drug and ligand complex and any of a peptidyl, hydrazine, or disulfide linker may attach a diagnostic agent to the complex. Other uses for additional linkers include linking analytical agents, biomolecules, targeting agents, and detectable labels to the drug-ligand complex.

Also within the scope of the present invention are compounds of the invention that are poly- or multi-valent species, including, for example, species such as dimers, trimers, tetramers and higher homologs of the compounds of the invention or reactive analogues thereof. The poly- and multi-valent species can be assembled from a single species or more than one species of the invention. For example, a dimeric construct can be "homo-dimeric" or

"heterodimeric." Moreover, poly- and multi-valent constructs in which a compound of the invention or a reactive analogue thereof, is attached to an oligomeric or polymeric framework (*e.g.*, polylysine, dextran, hydroxyethyl starch and the like) are within the scope of the present invention. The framework is preferably polyfunctional (*i.e.* having an array of reactive sites for attaching compounds of the invention). Moreover, the framework can be derivatized with a single species of the invention or more than one species of the invention.

Moreover, the present invention includes compounds that are functionalized to afford compounds having water-solubility that is enhanced relative to analogous compounds that are not similarly functionalized. Thus, any of the substituents set forth herein can be replaced with analogous radicals that have enhanced water solubility. For example, it is within the scope of the invention to, for example, replace a hydroxyl group with a diol, or an amine with a quaternary amine, hydroxy amine or similar more water-soluble moiety. In a preferred embodiment, additional water solubility is imparted by substitution at a site not essential for the activity towards the ion channel of the compounds set forth herein with a moiety that enhances the water solubility of the parent compounds. Methods of enhancing the water-solubility of organic compounds are known in the art. Such methods include, but are not limited to, functionalizing an organic nucleus with a permanently charged moiety, e.g., quaternary ammonium, or a group that is charged at a physiologically relevant pH, e.g. carboxylic acid, amine. Other methods include, appending to the organic nucleus hydroxyl- or amine-containing groups, e.g. alcohols, polyols, polyethers, and the like. Representative examples include, but are not limited to, polylysine, polyethyleneimine, poly(ethyleneglycol) and poly(propyleneglycol). Suitable functionalization chemistries and strategies for these compounds are known in the art. *See*, for example, Dunn, R.L., *et al.*, Eds. POLYMERIC DRUGS AND DRUG DELIVERY SYSTEMS, ACS Symposium Series Vol. 469, American Chemical Society, Washington, D.C. 1991.

DRUGS

Drugs, depicted as "D" herein, are provided in the current invention as part of a drug-ligand conjugate where the drug is linked to a ligand through either a peptidyl, hydrazine, or disulfide linker. The drug must possess a desired biological activity and contain a reactive functional group in order to link to the ligand. The desired biological activity includes the diagnosis, cure, mitigation, treatment, or prevention of disease in an animal such as a human.

Thus, so long as it has the needed reactive functional group, the term "drug" refers to chemicals recognized as drugs in the official United States Pharmacopeia, official Homeopathic Pharmacopeia of the United States, or official National Formulary, or any supplement thereof Exemplary drugs are set forth in the Physician's Desk Reference (PDR) and in the Orange Book maintained by the U.S. Food and Drug Administration (FDA). New drugs are being continually being discovered and developed, and the present invention provides that these new drugs may also be incorporated into the drug-ligand complex of the current invention.

Preferred functional groups include primary or secondary amines, hydroxyls, sulfhydryls, carboxyls, aldehydes, and ketones. More preferred functional groups include hydroxyls, primary or secondary amines, sulfhydryls and carboxylic acid functional groups. Even more preferred functional groups include hydroxyls, primary and secondary amines and carboxylic acid functional groups. The drug must have at least one, but may have 2, 3, 4, 5, 6 or more reactive functional groups. Additionally, a self-immolative spacer, L^1, may be incorporated between the reactive functional group of the drug and the peptide, hydrazine or disulfide linker.

The drug-ligand conjugate is effective for the usual purposes for which the corresponding drugs are effective, but have superior efficacy because of the ability, inherent in the ligand, to transport the drug to the desired cell where it is of particular benefit.

Exemplary drugs include proteins, peptides, and small molecule drugs containing a functional group for linkage to the ligand. More specifically, these drugs include, for example, the enzyme inhibitors such as dihydrofolate reductase inhibitors, and thymidylate synthase inhibitors, DNA intercalators, DNA cleavers, topoisomerase inhibitors, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diynenes, the podophyllotoxins, differentiation inducers, and taxols.

Preferred drugs of the current invention include cytotoxic drugs useful in cancer therapy and other small molecules, proteins or polypeptides with desired biological activity, such as a toxin. The drug may be selected to be activated at a tumor cells by conjugation to a tumor-specific ligand. These tumor specific drug-ligand conjugates have tumor specificity arising from the specificity of the ligand. Examples of this are drug-ligand conjugates that are highly selective substrates for tumor specific enzymes, where these enzymes are present in the proximity of the tumor in sufficient amounts to generate cytotoxic levels of free drug in the

vicinity of the tumor. One advantage of these tumor-specific drug-ligand complexes is that they are stable to adventitious proteases in the human serum. Another advantage of the drug-ligand complex is that they are less toxic than the corresponding free drug; additionally, the specificity of the complex may allow for lower overall concentrations to be used relative to the free drug since the increased specificity will result in a higher percentage of the complex to be present at the tumor site.

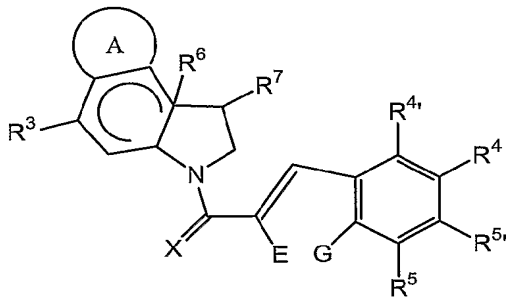
Cytotoxins

Cytotoxic drugs useful in the current invention include, for example, duocarmycins and CC-1065, and analogues thereof, including CBI (1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indol-4-one)-based analogues, MCBI (7-methoxy-1,2,9,9a-tetra-hydrocyclopropa[c]benz[e]indol-4-one)-based analogues and CCBI (7-cyano-1,2,9,9a-tetra-hydrocyclo-propa[c]benz[e]-indol-4-one)-based analogues of the duocarmycins and CC-1065, doxorubicin and doxorubicin conjugates such as morpholino-doxorubicin and cyanomorpholino-doxorubicin, dolastatins such as dolestatin-10, combretastatin, calicheamicin, maytansine, maytansine analogs, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones , Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin and daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin and podophyllotoxin derivatives such as etoposide or etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 -acetyl spermidine, camptothecin, and their analogues. Other known drugs may be modified in order to provide a functional group for conjugation to the linker described herein. Such chemical modification is known in the art.

Preferred cytotoxins for use in the current invention include: duocarmycins and CC-1065, and CCBI-based and MCBI-based analogues thereof, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatin-10, combretastatin, calicheamicin, maytansine, DM-1, auristatin E, AEB, AEFP, MMAE, Tubulysin A, Disorazole, epothilone A and epothilone B.

Particularly preferred cytotoxins of the present invention are active, potent duocarmycin derivatives and CC-1065. The parent agents are exceptionally potent antitumor antibiotics that derive their biological effects through the reversible, stereoelectronically controlled sequence selective alkylation of DNA (Boger *et al. J. Org. Chem.* **55**: 4499 (1990); Boger *et al. J. Am. Chem. Soc.* **112**: 8961 (1990); Boger *et al., J. Am. Chem. Soc.* **113**: 6645 (1991); Boger *et al. J. Am. Chem. Soc.* **115**: 9872 (1993); Boger *et al., Bioorg. Med. Chem. Lett.* **2**: 759 (1992)). Subsequent to the initial disclosure of the duocarmycins, extensive efforts have been devoted to elucidating the DNA alkylation selectivity of the duocarmycins and its structural origin.

A particularly preferred aspect of the current invention provides a cytotoxic compound having a structure according to Formula 7:



(7)

in which ring system A is a member selected from substituted or unsubstituted aryl substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups. Exemplary ring systems include phenyl and pyrrole.

The symbols E and G are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond or E and G are optionally joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl.

The symbol X represents a member selected from O, S and NR^{23}. R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

The symbol R^3 represents a member selected from (=O), SR^{11}, NHR^{11} and OR^{11}, in which R^{11} is H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl,

diphosphates, triphosphates, acyl, $C(O)R^{12}$ R^{13}, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, $C(O)CHR^{12}R^{13}$, SR^{12} or $SiR^{12}R^{13}R^{14}$. The symbols R^{12}, R^{13}, and R^{14} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. One or more of R^{12}, R^{13}, or R^{14} can include a cleaveable group within its structure.

R^4, $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$, wherein n is an integer from 1 to 20. R^{15} and R^{16} independently represent H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms. One exemplarly structure is aniline.

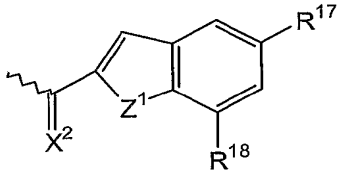
R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} optionally contain one or more cleaveable groups within their structure. Exemplary cleaveable groups include, but are not limited to peptides, amino acids, hydrazines, and disulfides.

At least one of R^{11}, R^{12}, R^{13}, R^{15} and R^{16} is used to join the drug to a linker of the present invention, as described herein, for example to L^1, if present or to F, H, or J.

In a still further exemplary embodiment, at least one of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} bears a reactive group appropriate for conjugating the compound. In a further exemplary embodiment, R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} are independently selected from H, substituted alkyl and substituted heteroalkyl and have a reactive functional group at the free terminus of the alkyl or heteroalkyl moiety. One or more of R^4, $R^{4'}$, R^5, $R^{5'}$, R^{11}, R^{12}, R^{13}, R^{15} and R^{16} may be conjugated to another species, *e.g*, targeting agent, detectable label, solid support, etc.

As will be apparent from the discussion herein, when at least one of R^{15} and R^{16} comprises a reactive functional group, that group can be a component of a bond between the drug and another molecule. In an exemplary embodiment in which at least one of R^{15} and R^{16} comprises a linkage between the drug and another species, at least one of R^{15} and R^{16} is a moiety that is cleaved by an enzyme.

In a further exemplary embodiment, at least one of R^4, $R^{4'}$, R^5 and $R^{5'}$ is:



(8).

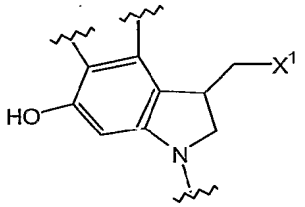
In Formula 8, the symbols X^2 and Z^1 represent members independently selected from O, S and NR^{23}. The groups R^{17} and R^{18} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{19}R^{20}$, $NC(O)R^{19}$, $OC(O)NR^{19}$, $OC(O)OR^{19}$, $C(O)R^{19}$, SR^{19} or OR^{19}, with the proviso that at least one one of R^{12}, R^{13}, R^{19}, or R^{20} comprises a linker of the present invention, as disclosed herein.

The symbols R^{19} and R^{20} independently represent substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, substituted or unsubstituted peptidyl, wherein R^{19} and R^{20} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms, with the proviso that when Z^1 is NH, both R^{17} and R^{18} are not H, and R^{17} is not NH_2. Throughout the present specification, the symbols R^{19} and R^{20} also encompass the groups set forth for R^4 and R^5. Thus, for example, it is within the scope of the present invention to provide compounds having two or more of the fused phenyl-heterocyclic ring systems set forth immediately above linked in series, or a fused ring in combination with a linker. Moreover, in those embodiments in which a linker is present, the linker may be present as an R^4, $R^{4'}$, R^5, or $R^{5'}$ substituent or as an R^{17} or R^{18} substituent.

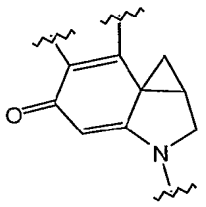
R^6 is a single bond which is either present or absent. When R^6 is present, R^6 and R^7 are joined to form a cyclopropyl ring. R^7 is CH_2-X^1 or $-CH_2-$. When R^7 is $-CH_2-$ it is a

component of the cyclopropane ring. The symbol X^1 represents a leaving group such as a halogen, for example Cl, Br or F. The combinations of R^6 and R^7 are interpreted in a manner that does not violate the principles of chemical valence.

The curved line within the six-membered ring indicates that the ring may have one or more degree of unsaturation, and it may be aromatic. Thus, ring structures such as those set forth below, and related structures, are within the scope of Formula (9):



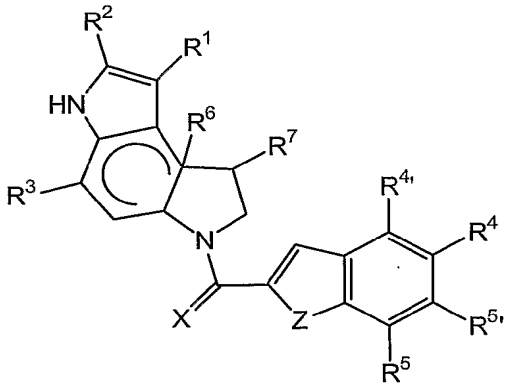
and



(9).

In an exemplary embodiment, ring system A is a substituted or unsubstituted phenyl ring. Ring system A is preferably substituted with one or more aryl group substituents as set forth in the definitions section herein. In one preferred embodiment, the phenyl ring is substituted with a CN or methoxy moiety.

In another exemplary embodiment, the invention provides a compound having a structure according to Formula 10:



(10).

In this embodiment, the identities of the radicals R^3, R^4, $R^{4'}$, R^5, $R^{5'}$, R^6, R^7 and X are substantially as described above. The symbol Z is a member independently selected from O, S and NR^{23}. The symbol R^{23} represents a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl. Each R^{23} is independently selected. The symbol R^1 represents H, substituted or unsubstituted lower alkyl, or $C(O)R^8$ or CO_2R^8. R^8 is a member selected from substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10} and OR^9. R^9,

and R^{10} are independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl. The radical R^2 is H, or substituted or unsubstituted lower alkyl. It is generally preferred that when R^2 is substituted alkyl, it is other than a perfluoroalkyl, e.g., CF_3. In one embodiment, R^2 is a substituted alkyl wherein the substitution is not a halogen. In another embodiment, R^2 is an unsubstituted alkyl.

As discussed above, X^1 may be a leaving group. Useful leaving groups include, but are not limited to, halogens, azides, sulfonic esters (e.g., alkylsulfonyl, arylsulfonyl), oxonium ions, alkyl perchlorates, ammonioalkanesulfonate esters, alkylfluorosulfonates and fluorinated compounds (*e.g.*, triflates, nonaflates, tresylates) and the like. Particular halogens useful as leaving groups are F, Cl and Br. The choice of these and other leaving groups appropriate for a particular set of reaction conditions is within the abilities of those of skill in the art (*see*, for example, March J, ADVANCED ORGANIC CHEMISTRY, 2nd Edition, John Wiley and Sons, 1992; Sandler SR, Karo W, ORGANIC FUNCTIONAL GROUP PREPARATIONS, 2nd Edition, Academic Press, Inc., 1983; and Wade LG, COMPENDIUM OF ORGANIC SYNTHETIC METHODS, John Wiley and Sons, 1980).

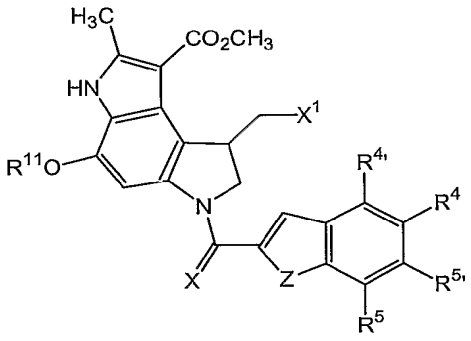
In an exemplary embodiment R^1 is an ester moiety, such as CO_2CH_3. In a further exemplary embodiment, R^2 is a lower alkyl group, which may be substituted or unsubstituted. A presently preferred lower alkyl group is CH_3. In a still further embodiment, R^1 is CO_2CH_3, and R^2 is CH_3.

In yet another exemplary embodiment, R^4, $R^{4'}$, R^5, and $R^{5'}$ are members independently selected from H, halogen, NH_2, OMe, $O(CH_2)_2N(Me)_2$ and NO_2.

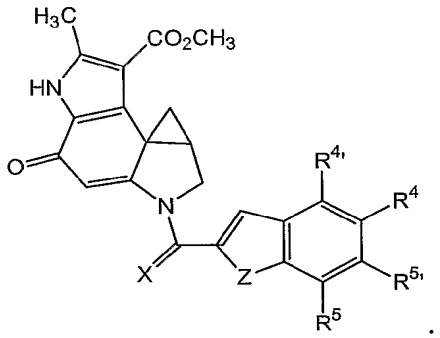
In one embodiment, the drug is selected such that the leaving group X^1 is a member selected from the group consisting of halogen, alkylsulfonyl, arylsulfonyl, and azide. In another embodiment, Z is O. In certain embodiments, R^1 may be CO_2CH_3 or R^2 may be CH_3; additionally, R^1 may be CO_2CH_3, and R^2 may be CH_3. One of R^4, $R^{4'}$, R^5 or $R^{5'}$ may be $C(O)R^{15}$ and the other three of R^4, $R^{4'}$, R^5 and $R^{5'}$ are H. Additionally, at least one of R^4, $R^{4'}$, R^5 and $R^{5'}$ may be other than a member selected from H and OCH_3. In one embodiment, R^4, $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from H, halogen, NH_2, $O(CH_2)_2N(Me)_2$ and NO_2.

In a preferred embodiment, one of R^4, $R^{4'}$, R^5 or $R^{5'}$ is $O(CH_2)_2N(Me)_2$ and the others of R^4, $R^{4'}$, R^5 and $R^{5'}$ are H. In another embodiment, R^7 is CH_2-X^1 where X^1 is F, Cl or Br and R^6 is absent.

In yet another exemplary embodiment, the invention provides compounds having a structure according to Formula 11 and 12:



and

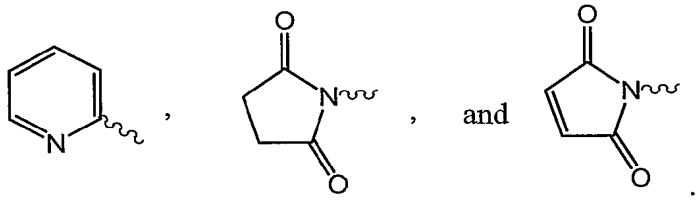


(11) (12)

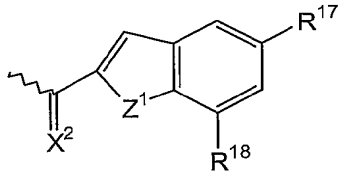
In one embodiment of the Formula above, X is preferably O; and Z is preferably O. In another embodiment, Z is NR^{23} or O. Alternatively, one of R^4, $R^{4'}$, R^5 or $R^{5'}$ may be $O(CH_2)_2N(Me)_2$ while the other three of R^4, $R^{4'}$, R^5 or $R^{5'}$ are H. In one embodiment, R^4, $R^{4'}$, R^5 or $R^{5'}$ may be selected from the group consisting of R^{29}, $COOR^{29}$, $C(O)NR^{29}$, and $C(O)NNR^{29}$, wherein R^{29} is selected from the group consisting of H, OH, substituted alkyl, unsubstituted alkyl, substituted cycloalkyl, unsubstituted cycloalkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted cycloheteroalkyl , unsubstituted cycloheteroalkyl, substituted heteroaryl, and unsubstituted heteroaryl.

In another embodiment of the Formula above X is preferably O, Z is preferably O, R_1 is preferably CO_2CH_3, R_7 is preferably CH_2-Cl, R_2 is preferably CH_3, R_3 is preferably OH. Alternatively, one of R^4, $R^{4'}$, R^5 or $R^{5'}$ may be $NHC(O)(C_6H_4)NH_2$ while the other three of R^4, $R^{4'}$, R^5 or $R^{5'}$ are H.

In one embodiment, R^{29} may be selected from the group consisting of:



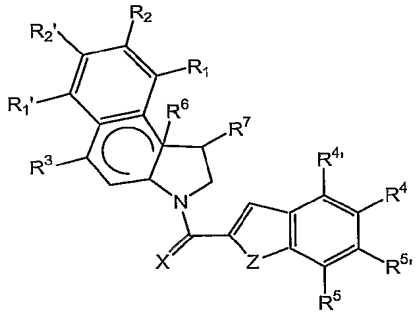
In yet another embodiment of the drug, one member selected from R^4 and R^5 is:



wherein X^2 and Z^1 are members independently selected from O, S and NR^{23}; R^{17} and R^{18} are members independently selected from the group consisting of H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{19}R^{20}$, $NC(O)R^{19}$, $OC(O)NR^{19}$, $OC(O)OR^{19}$, $C(O)R^{19}$, OR^{19}, and $O(CH_2)_nN(CH_3)_2$. In this embodiment, n is an integer from 1 to 20; R^{19} and R^{20} are independently selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted heterocycloalkyl, wherein R^{19} and R^{20} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms, wherein one of R^{11}, R^{12}, R^{13}, R^{15}, R^{16}, R^{19}, or R^{20} links said drug to L^1, if present, or to F. In one preferred embodiment, X^2 is O and Z^1 is O or NR^{23}.

Another preferred structure of the duocarmycin analog of Formula 7 is a structure in which the ring system A is an unsubstituted or substituted phenyl ring. The preferred substituents on the drug molecule described hereinabove for the structure of Formula 7 when the ring system A is a pyrrole are also preferred substituents when the ring system A is an unsubstituted or substituted phenyl ring.

For example, in a preferred embodiment, the drug (D) comprises a structure:



In this structure, R^3, R^6, R^7, X are as described above for Formula 7. Furthermore, Z is a member selected from O, S and NR^{23}, wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

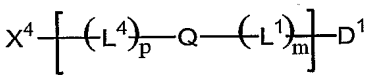
$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9, in which R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and $R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl.

At least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links the drug to L^1, if present, or to F, H, or J.

In a preferred embodiment, one of R^4, $R^{4'}$, R^5 or $R^{5'}$ is $O(CH_2)_2N(Me)_2$ and the others of R^4, $R^{4'}$, R^5 and $R^{5'}$ are H. In another embodiment, R^7 is CH_2-X^1 where X^1 is F, Cl or Br and R^6 is absent.

In one embodiment, the invention provides a cytotoxic drug-ligand compound having a structure according to the following formula:



wherein the symbol L^1 represents a self-immolative spacer where m is an integer of 0, 1, 2, 3, 4, 5, or 6.

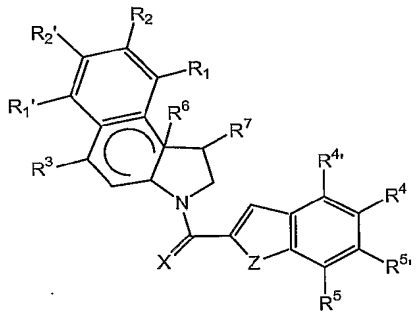
The symbol X^4 represents a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents.

The symbol L^4 represents a linker member, and p is 0 or 1. L^4 is a moiety that imparts increased solubility or decreased aggregation properties to the conjugates. Examples of L^4 moieties include substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl,

substituted heteroalkyl, or unsubstituted heteroalkyl, any of which may be straight, branched, or cyclic, a positively or negatively charged amino acid polymer, such as polylysine or polyargenine, or other polymers such as polyethylene glycol.

The symbol Q represent any cleavable linker including, but not limited to, any of the peptidyl, hydrozone, and disulfide linkers described herein. Other suitable linkers include, but are not limited to, those described in U.S. Patent No. 6,214,345; U.S. Patent Applications Publication Nos. 2003/0096743, 2003/0130189, and 2004/121940; PCT Patent Applications Publication Nos. WO 03/026577 and WO 04/043493; and European Patent Applications Publication Nos. EP1243276 and EP1370298, all of which are incorporated herein by reference. Cleavable linkers include those that can be selectively cleaved by a chemical or biological process and upon cleavage separate the drug, D^1, from X^4. Cleavage can occur anywhere along the length of the linker or at either terminus of the linker.

The symbol D^1 represents a drug having the following formula:



wherein X and Z are members independently selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8,

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$,

wherein R^8 is a member selected from NR^9R^{10} and OR^9 and R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy;

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

R^3 is a member selected from the group consisting of SR^{11}, NHR^{11} and OR^{11}, wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$, in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of R^{11}, R^{12}, and R^{13} links said drug to L^1, if present, or to Q,

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2$- joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group,

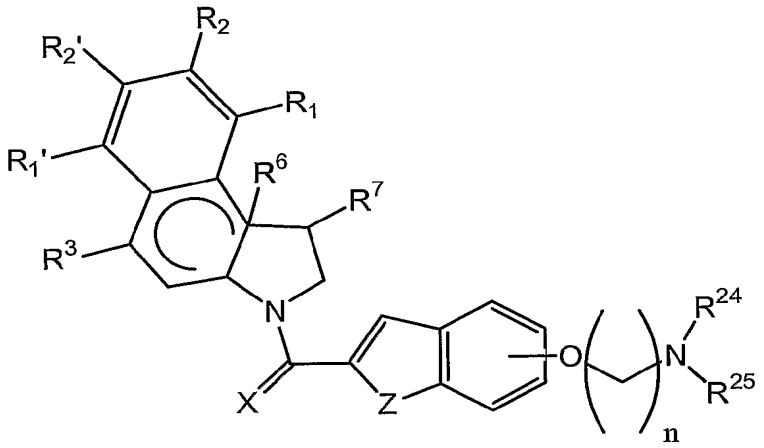
R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nNR^{24}R^{25}$ wherein n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

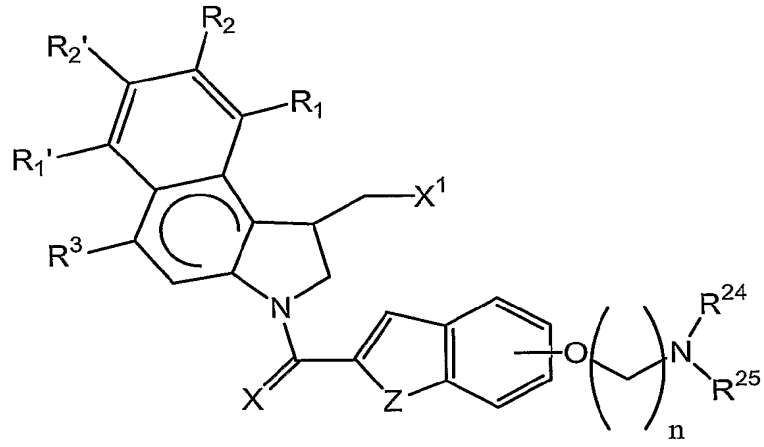
and R^{24} and R^{25} are independently selected from unsubstituted alkyl, and wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ is $O(CH_2)_nNR^{24}R^{25}$.

In some embodiments, n is 2. In some embodiments, R^{24} and R^{25} are methyl. In some embodiments, R^4 is $O(CH_2)_nNR^{24}R^{25}$ and $R^{4'}$, R^5 and $R^{5'}$ are H. In some embodiments, R^4 is $O(CH_2)_2N(CH_3)_2$ and $R^{4'}$, R^5 and $R^{5'}$ are H. In some embodiment, Q is a linker selected from F, H, and J, as described above. In some embodiments, R^1, $R^{1'}$, R^2, and $R^{2'}$ are H.

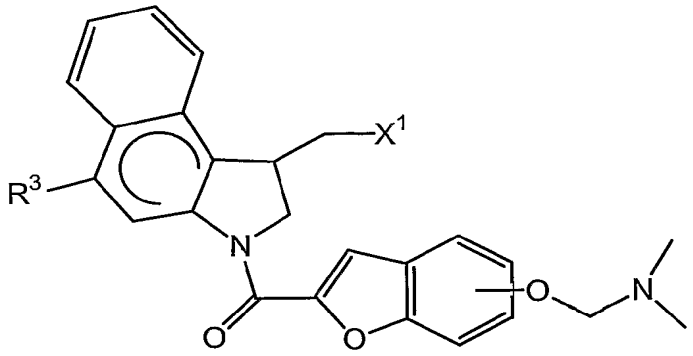
A preferred formula for drug, D^1, is the following:

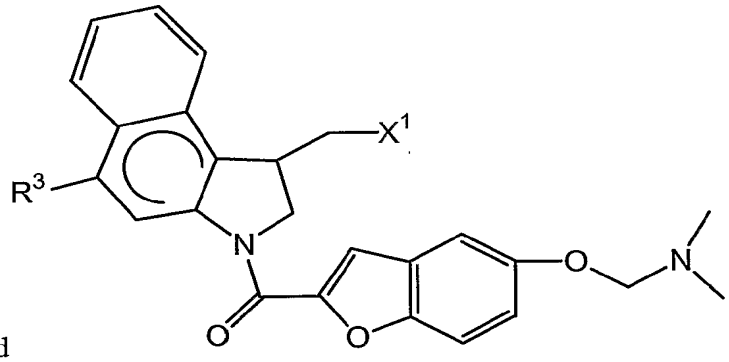


Another preferred embodiment of drug D^1 is the following:



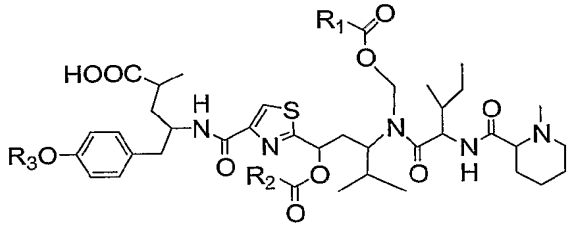
Yet additional preferred embodiments of drug D^1 are the following:





and

In another exemplary embodiment of the current invention, the cytotoxic drug may by a tubulysin analog or related compound, such as the compounds described by the structure according to Formula 13:



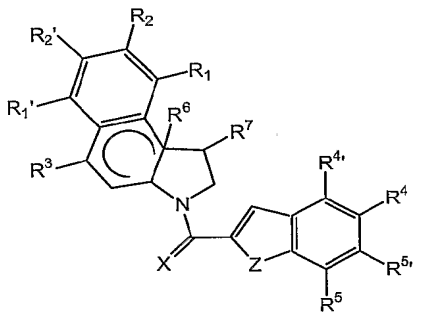
(13).

where R_1 and R_2 are H or a lower alkyl, or are more particularly isobutyl, ethyl, propyl, or *t*-butyl and R_3 is H or OH. Tubulysin and its use in treating cancer has been described in, for example, PCT Publications WO 2004/005327 and WO 2004/005326. The production of tubulysin compounds is described in DE10008089. Methods that may be used to link the tubulysin to various linkers of the current invention are provided in the examples. Preferred tubulysin analogs are Tubulysin A-F.

CBI Analogues

These particular compounds are CBI analogues in that they incorporate the 1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indole-4-one (CBI) alkylating domain or alkylation subunit. The compounds may be used as drugs. Preferred drugs of the current invention include cytotoxic drugs useful in cancer therapy. These compounds may or may not be conjugated or include linkers as described above. Cytotoxic drugs useful in the current invention include, for example, CBI (1,2,9,9a-tetrahydrocyclopropa[c]benz[e]indol-4-one)-based analogues, MCBI (7-methoxy-1,2,9,9a-tetra-hydrocyclopropa[c]benz[e]indol-4-one)-based analogues and CCBI (7-cyano-1,2,9,9a-tetra-hydrocyclo-propa[c]benz[e]-indol-4-one)-based analogues.

In one embodiment, a compound of the invention has the following formula (14):



(14)

wherein X and Z are independently selected from O, S and NR^{23} wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8,

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$,

each R^8 is a member independently selected from NR^9R^{10} and OR^9 and R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, substituted or unsubstituted lower alkyl, unsubstituted heteroalkyl, cyano, or alkoxy;

$R^{2'}$ is H, substituted or unsubstituted lower alkyl, or unsubstituted heteroalkyl,

R^3 is a member selected from the group consisting of SR^{11}, NHR^{11} and OR^{11}, wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$,

$C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$, in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, or R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2-$ joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group,

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nNR^{24}R^{25}$ wherein n is an integer from 1 to 20, preferably, n is an integer from 2 to 6;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

and R^{24} and R^{25} are independently selected from unsubstituted alkyl, and

wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ is $O(CH_2)_nNR^{24}R^{25}$.

As discussed above, X^1 may be a leaving group. Useful leaving groups include, but are not limited to, halogens, azides, sulfonic esters (e.g., alkylsulfonyl, arylsulfonyl), oxonium ions, alkyl perchlorates, ammonioalkanesulfonate esters, alkylfluorosulfonates and fluorinated compounds (*e.g.*, triflates, nonaflates, tresylates) and the like. Particular halogens useful as leaving groups are F, Cl and Br. The choice of these and other leaving groups appropriate for a particular set of reaction conditions is within the abilities of those of skill in the art (*see*, for example, March J, ADVANCED ORGANIC CHEMISTRY, 2nd Edition, John Wiley and Sons, 1992; Sandler SR, Karo W, ORGANIC FUNCTIONAL GROUP PREPARATIONS, 2nd Edition, Academic

Press, Inc., 1983; and Wade LG, COMPENDIUM OF ORGANIC SYNTHETIC METHODS, John Wiley and Sons, 1980).

In some embodiments, R^4, $R^{4'}$, R^5, and $R^{5'}$ are members independently selected from H, halogen, NH_2, OMe, $O(CH_2)_2N(Me)_2$ and NO_2. In some embodiments, at least one of R^4, $R^{4'}$, R^5, and $R^{5'}$ is $O(CH_2)_2N(Me)_2$. In some embodiments, one of R^4, $R^{4'}$, R^5 or $R^{5'}$ is $O(CH_2)_2N(Me)_2$ and the others of R^4, $R^{4'}$, R^5 and $R^{5'}$ are H. In other embodiments, R^4 is $O(CH_2)_2N(Me)_2$ and $R^{4'}$, R^5 and $R^{5'}$ are H.

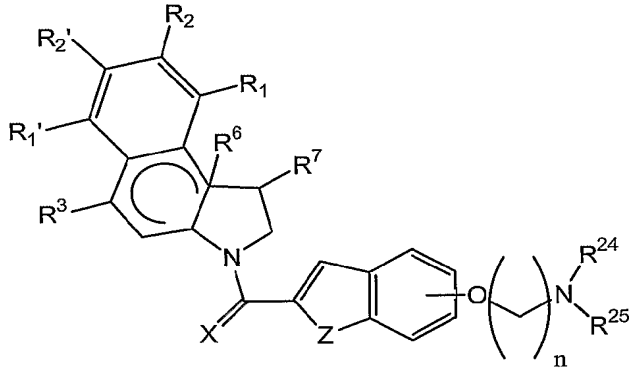
In some embodiments, R^7 is CH_2-X^1 where X^1 is F, Cl or Br and R^6 is absent. In some embodiments, the drug is selected such that the leaving group X^1 is a member selected from the group consisting of halogen, alkylsulfonyl, arylsulfonyl, and azide. In some embodiments, X^1 is Cl or Br.

In some embodiments, Z is O. In some embodiments, X and Z are O.

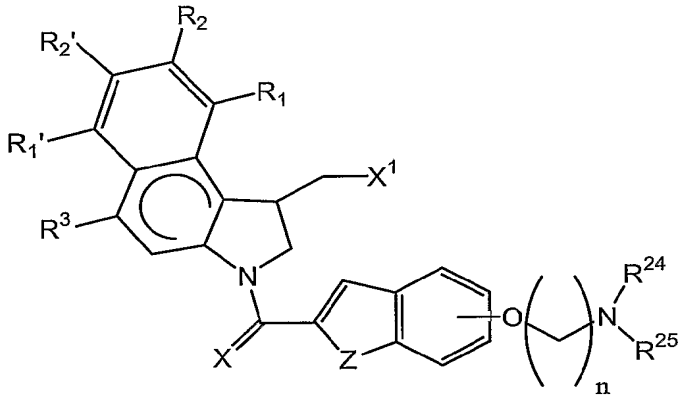
In some embodiments, R^2 is H, methyl, or cyano and R^1, $R^{1'}$, and $R^{2'}$ are H. In some embodiments, R^1, $R^{1'}$, R^2, and $R^{2'}$ are H. In some embodiments, R^1, $R^{1'}$, and $R^{2'}$ are H.

In some embodiments, R^3 is a reactive group as described below.

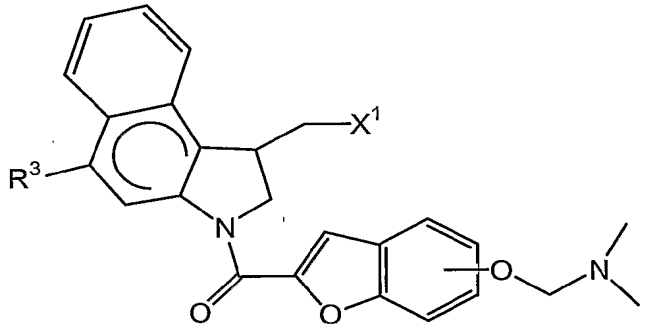
A preferred formula for the compound of Formula (14) is the following:

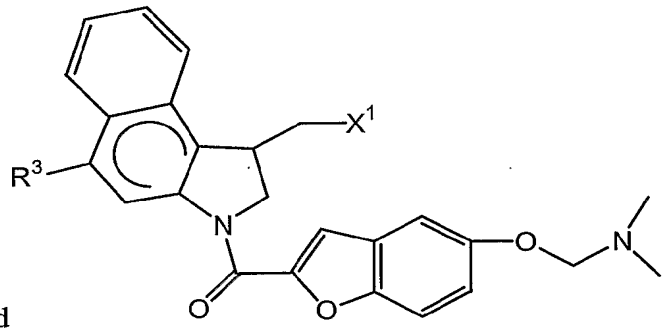


Another preferred embodiment for the compound of Formula (14) is the following:



Yet additional preferred embodiments for the compound of Formula (14) are the following:





and

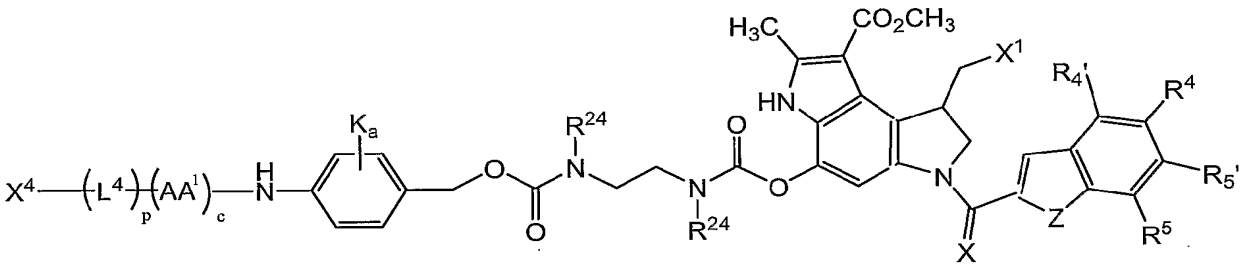
Preferred Duocarmycin and CBI Conjugates

The peptide, hydrazine or disulfide linkers of the invention can be used in conjugates containing duocarmycin or CBI analogs as cytotoxic agents. Preferred conjugates of the invention are described in further detail below. Unless otherwise indicated, substituents are

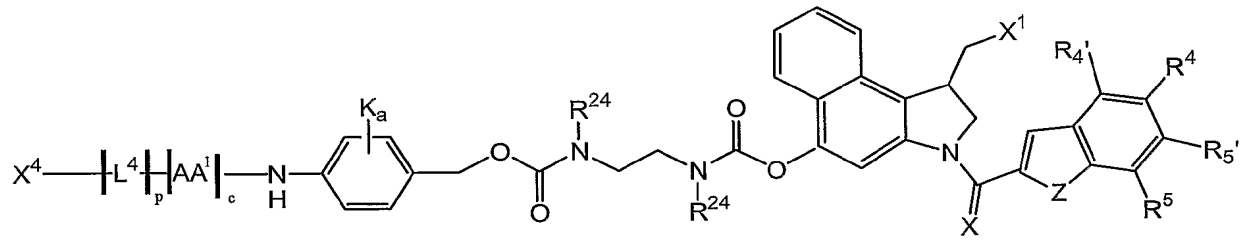
defined as set forth above in the sections regarding cytotoxins, peptide linkers, hydrazine linkers and disulfide linkers.

A. Peptide Linker Conjugates

In a preferred embodiment, the invention provides a peptide linker conjugate having the structure:



or



wherein X^1 is a halogen;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and

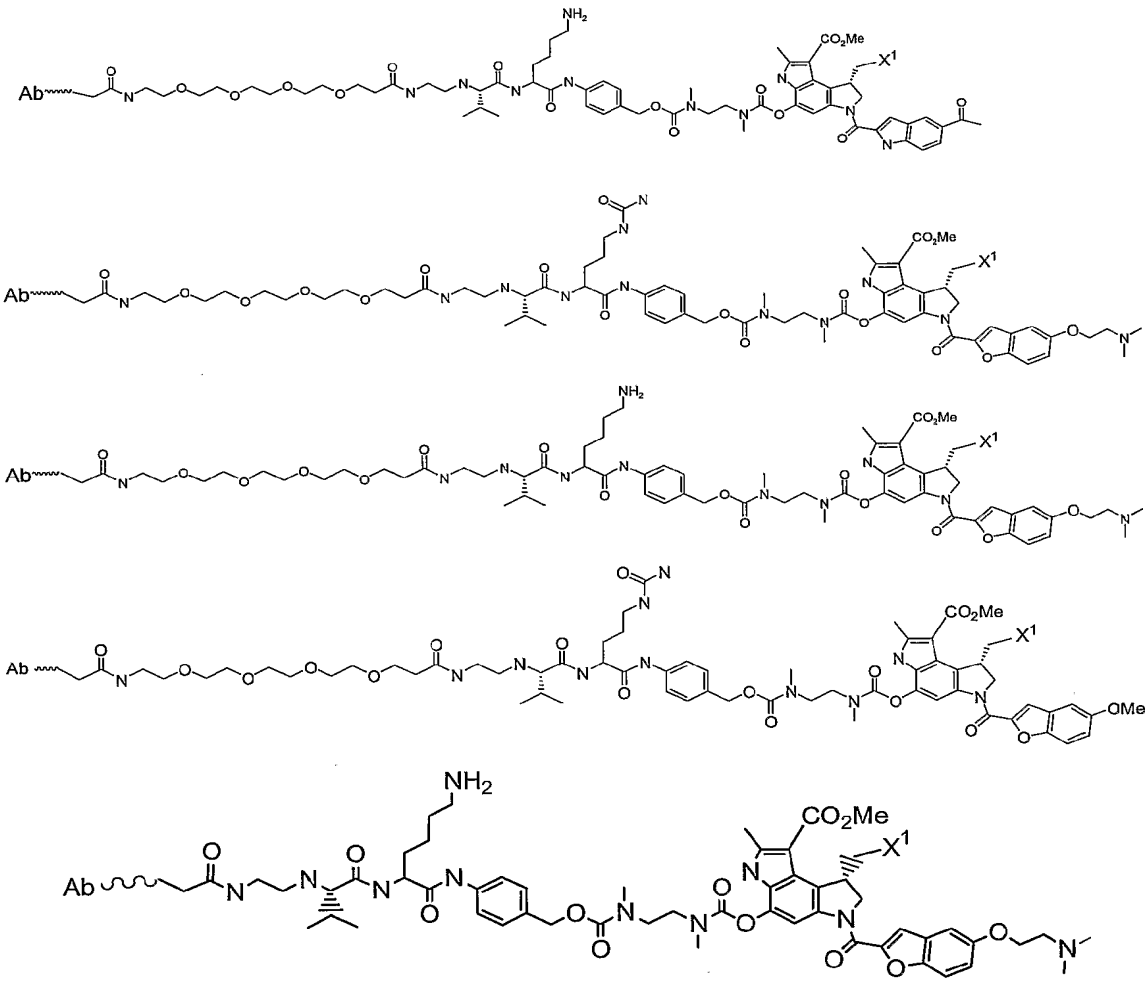
R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, OR^{15}, and $O(CH_2)_nN(CH_3)_2$

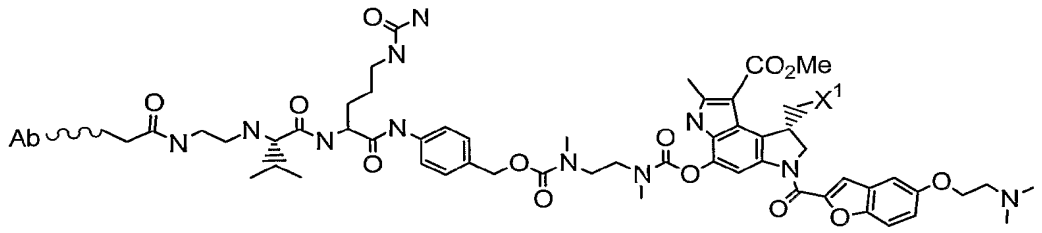
wherein

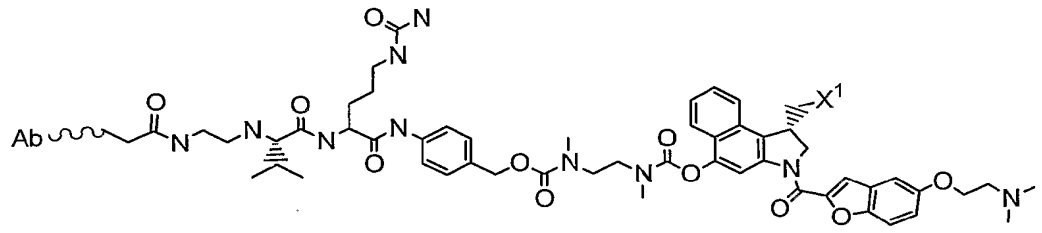
n is an integer from 1 to 20; and

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms.

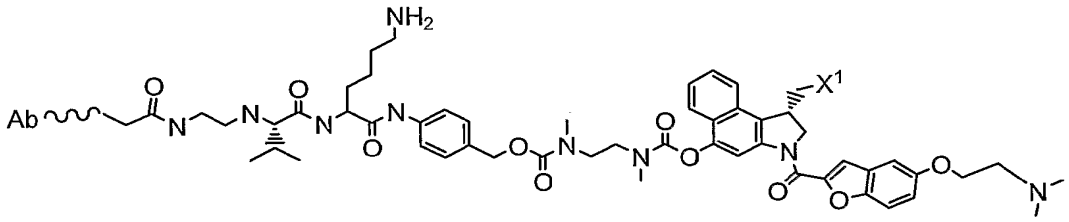
Non-limiting examples of such conjugates include the following structures:







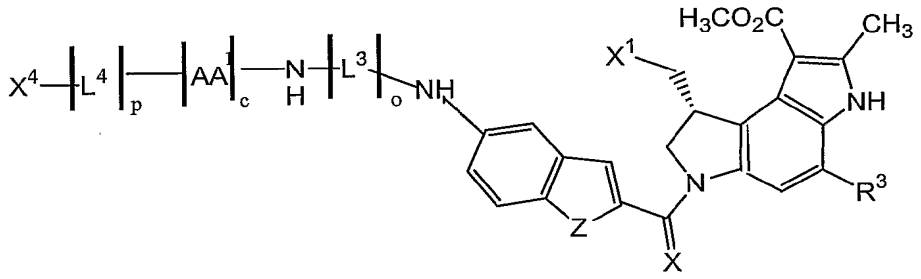
and



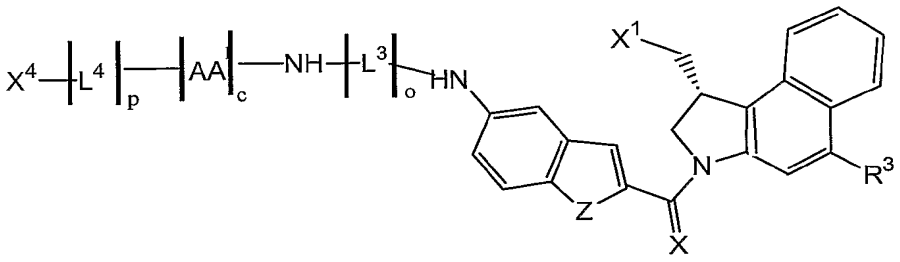
wherein X^1 is Cl or Br;

and wherein Ab is an antibody, or fragment thereof.

In another preferred embodiment, the invention provides a conjugate having the structure:



or



wherein X^1 is a leaving group;

Z and X are members independently selected from O, S and NR^{23},

wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and

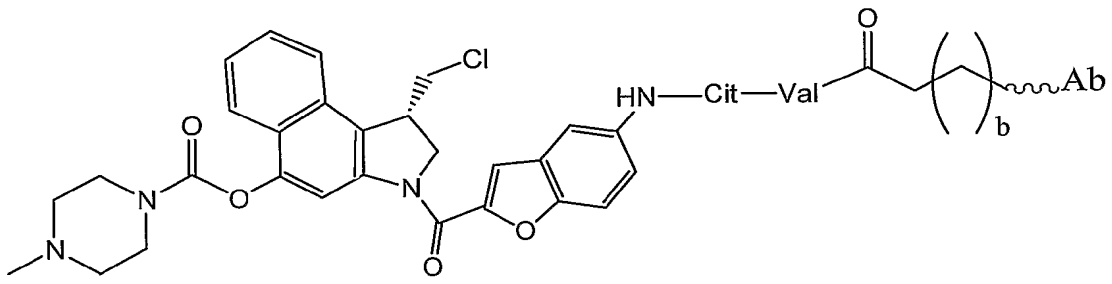
R^3 is selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, OR^{15}, and $O(CH_2)_nN(CH_3)_2$

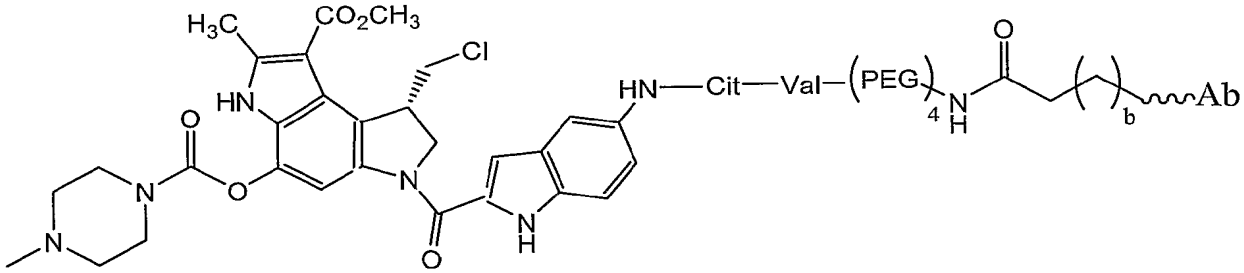
wherein

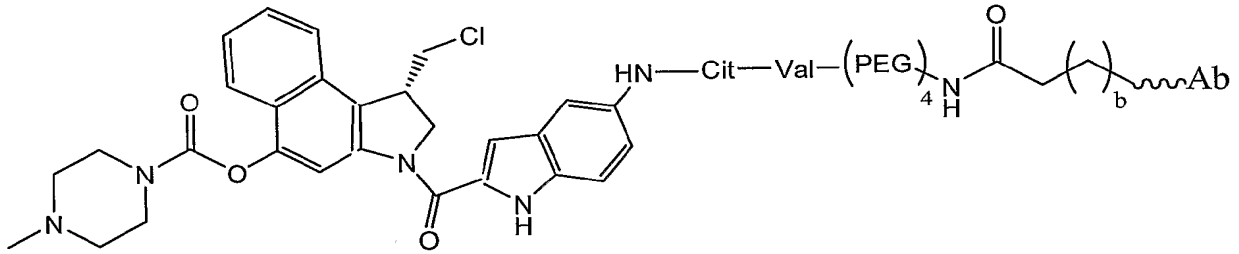
n is an integer from 1 to 20;

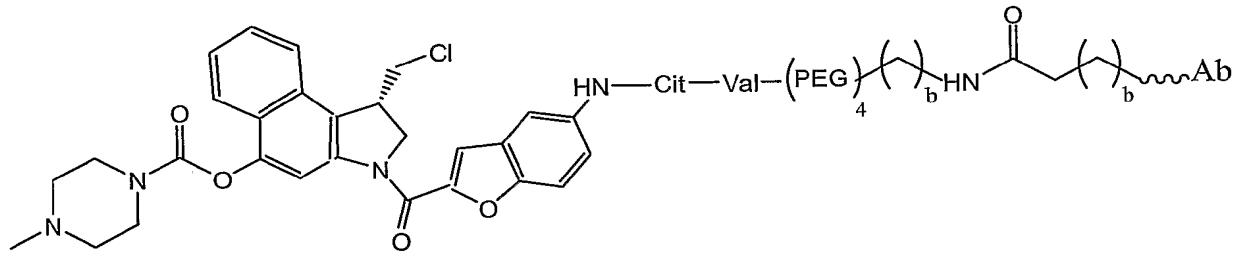
R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms.

Non-limiting examples of such conjugates include the following structures:

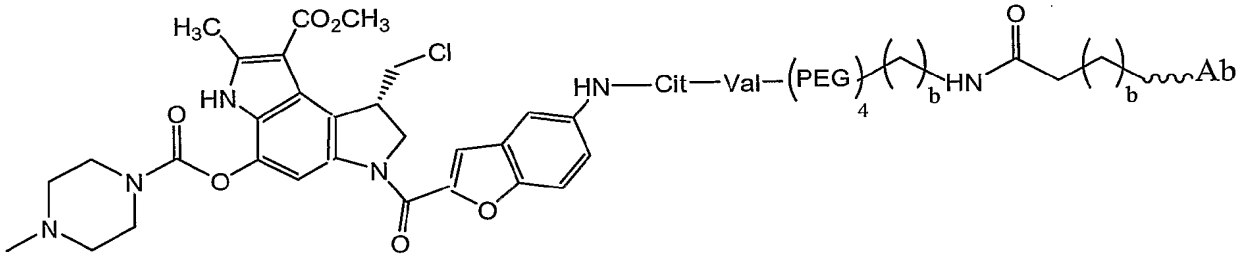






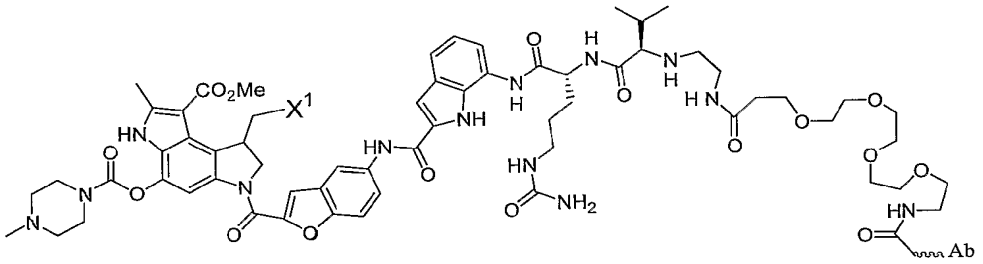


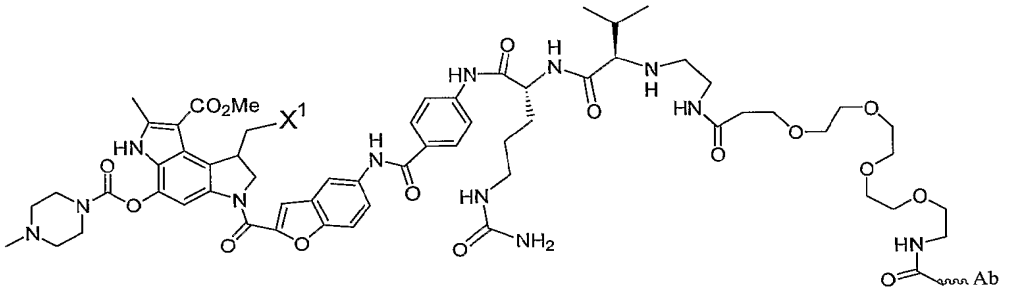
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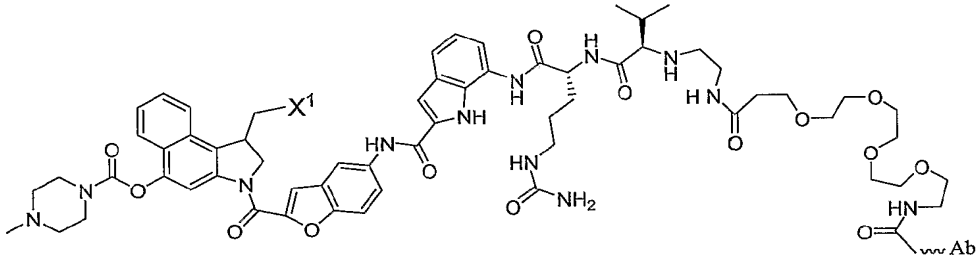


wherein each b is independently an integer from 0 to 20, and Ab is an antibody, or fragment thereof.

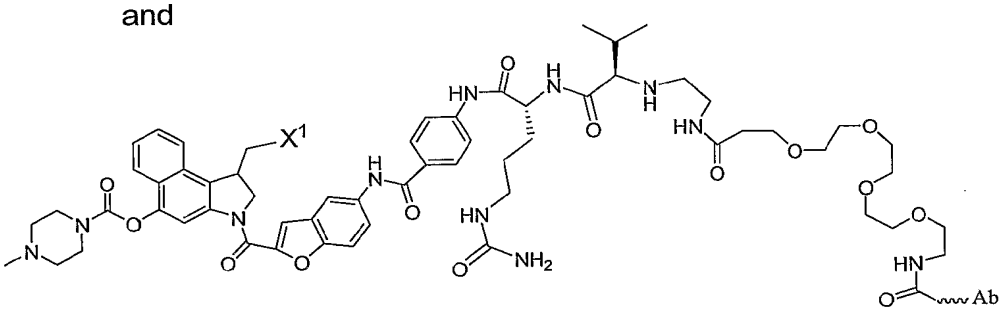
In yet other preferred embodiments, the invention provides a peptide linker conjugate selected from the following structures:





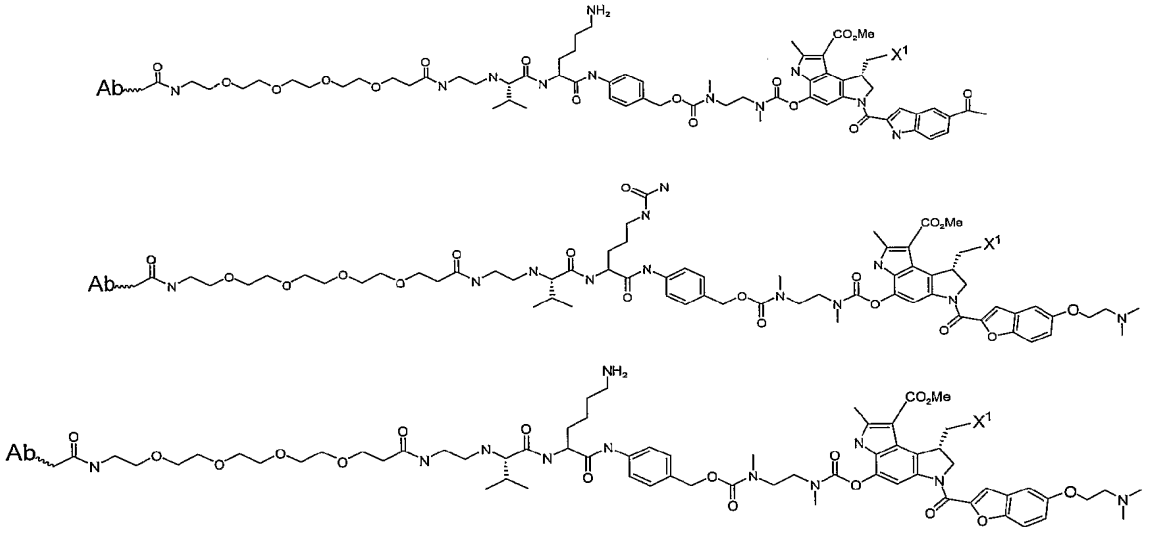


and

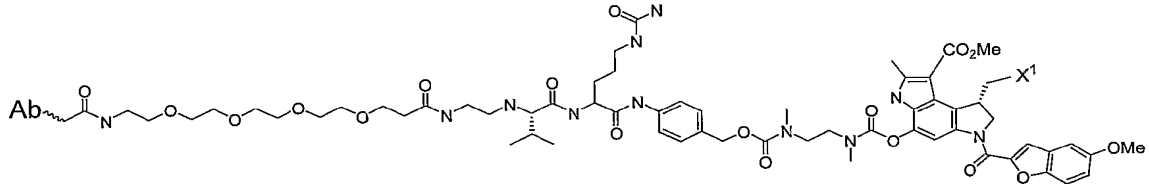


wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

In still other embodiments, the invention provides a peptide linker conjugate selected from the following structures:

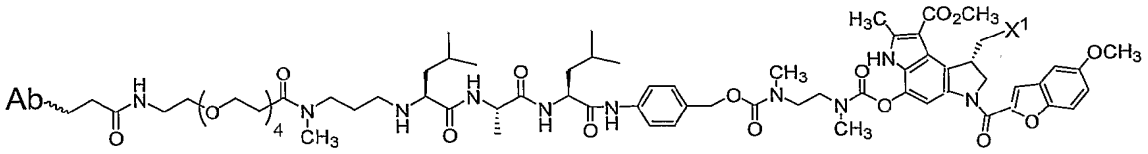


and



wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

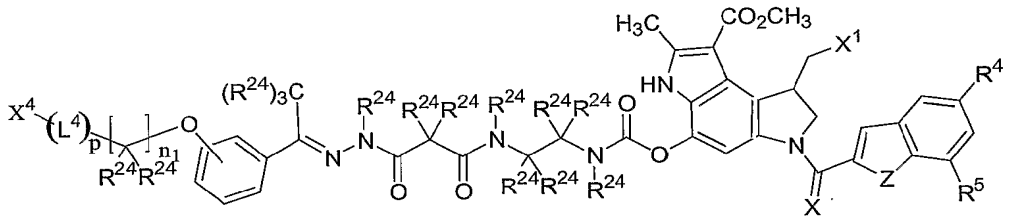
In still other embodiments, the invention provides a peptide linker conjugate having the following structure:



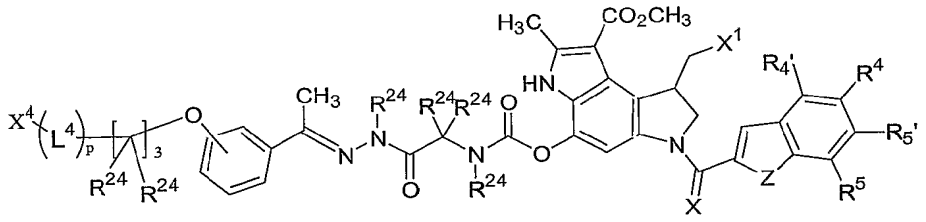
wherein X^1 is Cl or Br, and Ab is an antibody or fragment thereof.

B. Hydrazine Linker Conjugates

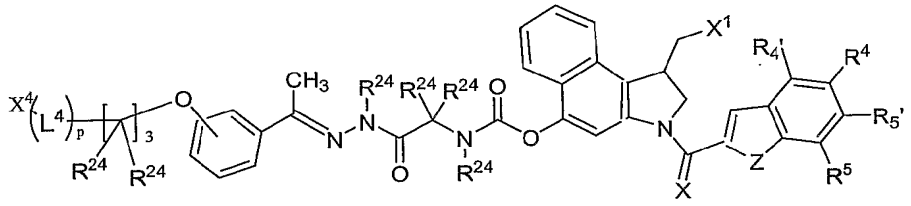
In a preferred embodiment, the invention provides a hydrazine linker conjugate having the structure:



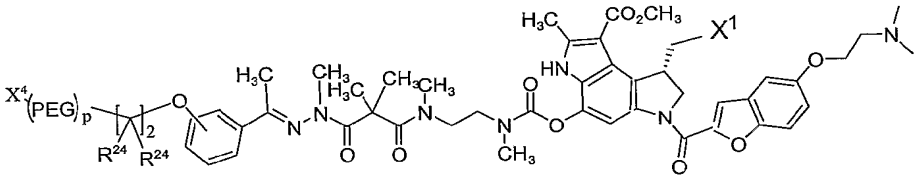
In another preferred embodiment, the invention provides a hydrazine linker conjugate having the structure:

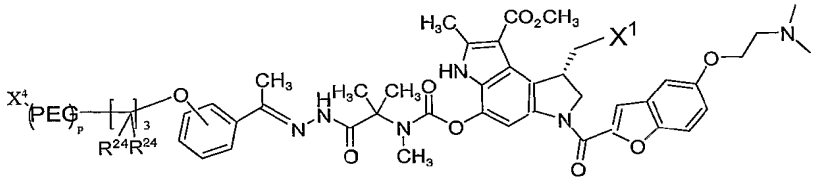


and

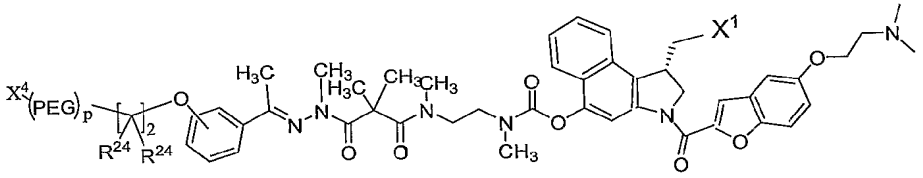


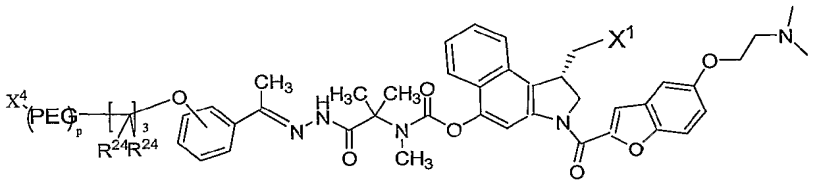
In yet other preferred embodiments, the invention provides a hydrazine linker conjugate having structure selected from:





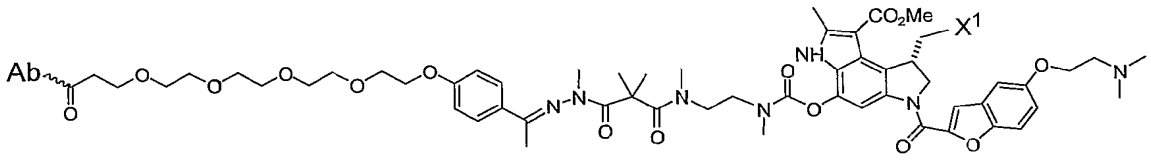
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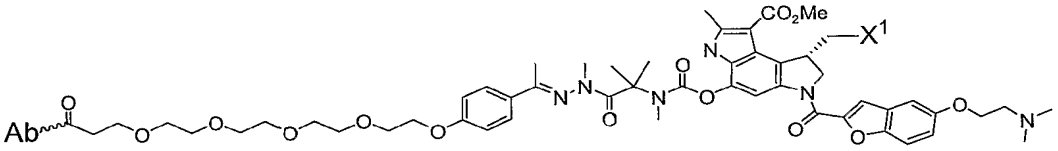


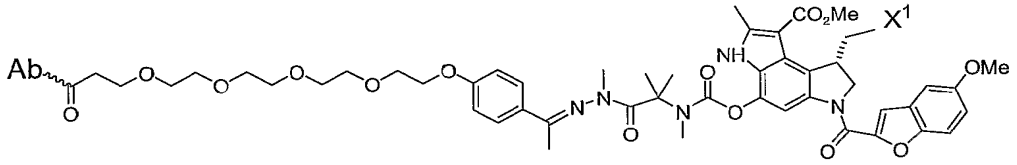


wherein PEG is a polyethylene glycol moiety and X^1 is Cl or Br.

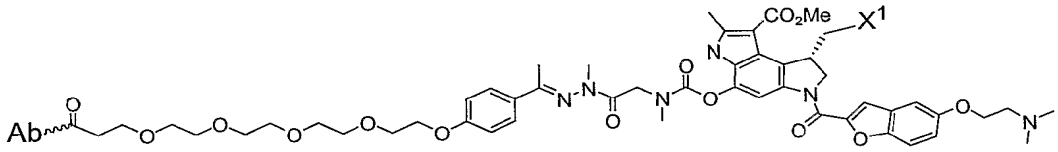
In still other preferred embodiments, the invention provides a hydrazine linker conjugate selected from the following structures:





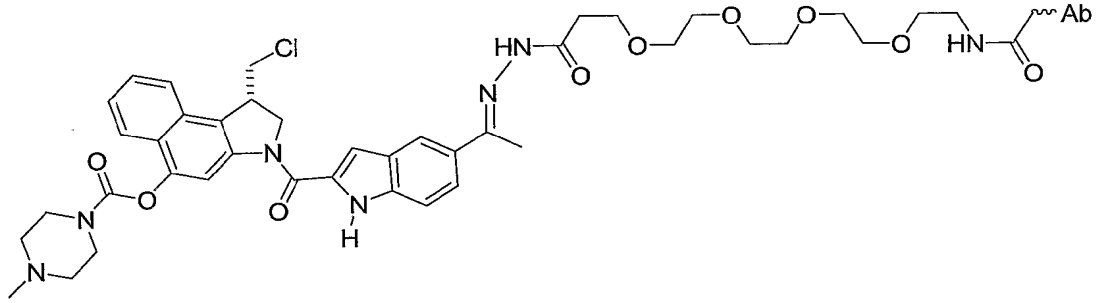


and

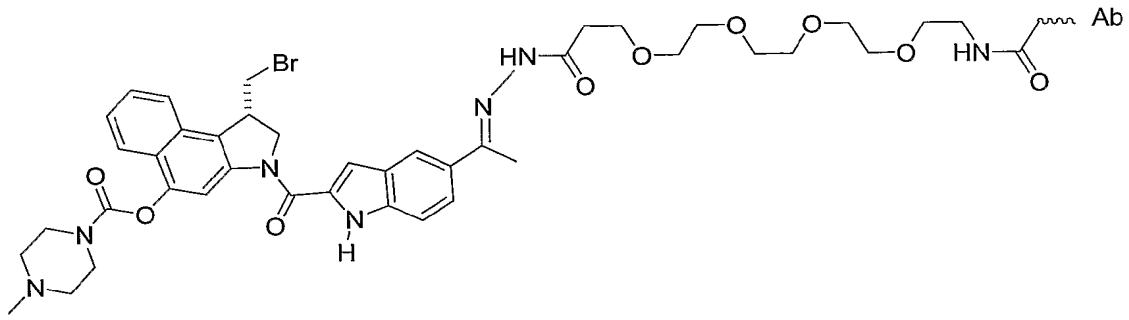


wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

In yet another preferred embodiment, there is a hydrazine linker conjugate selected from the following structures:

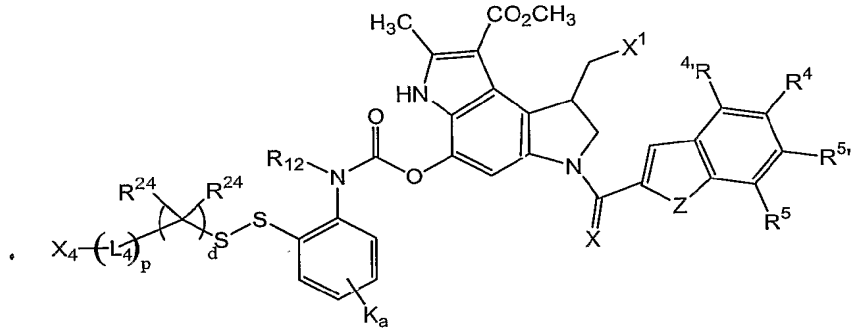


and

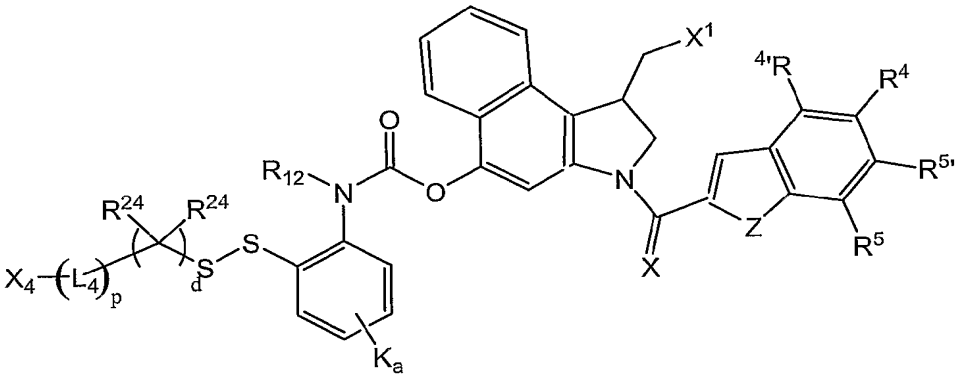


C. Disulfide Linker Conjugates

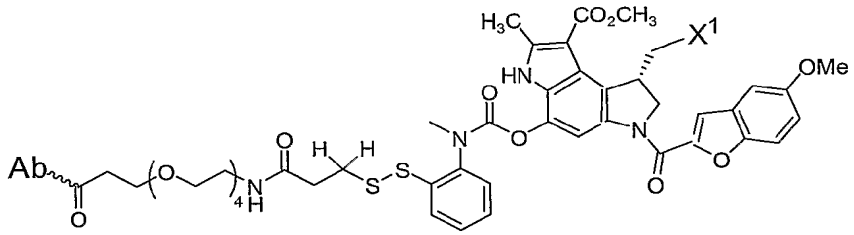
In a preferred embodiment, the invention provides a disulfide linker conjugate having the structure:



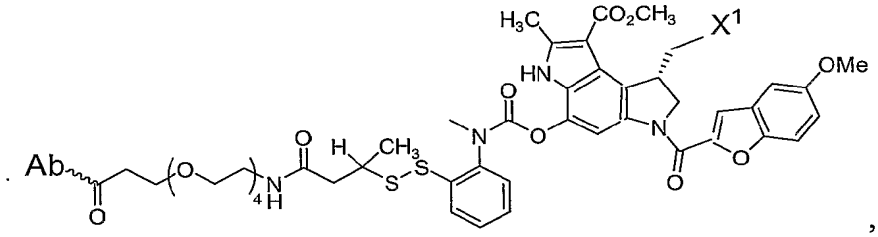
and



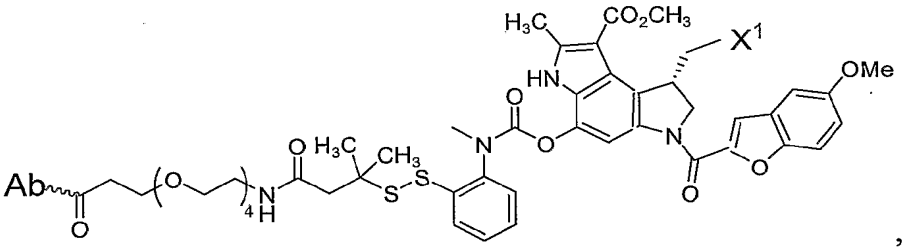
Non-limiting examples of such structures include the following:



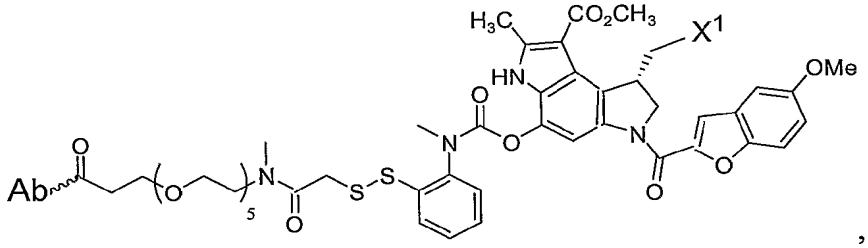
5



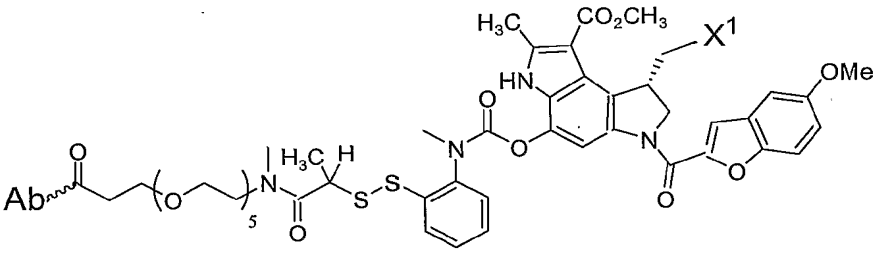
,



,

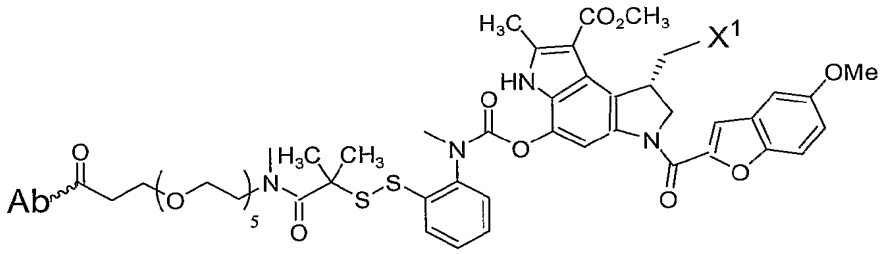


,



.

10 and

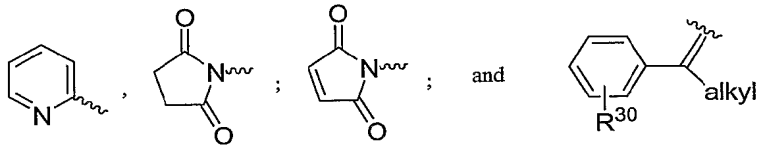


wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

LIGANDS

The ligands of the current invention are depicted as "X^4". In this invention, X^4 represents a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents. Preferred ligands are targeting agents, such as antibodies and fragments thereof.

In a preferred embodiment, the group X^4 can be described as a member selected from R^{29}, $COOR^{29}$, $C(O)NR^{29}$, and $C(O)NNR^{29}$ wherein R^{29} is a member selected from substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted heteroaryl. In yet another exemplary embodiment, R^{29} is a member selected from H; OH; $NHNH_2$;



wherein R^{30} represents substituted or unsubstituted alkyl terminated with a reactive functional group, substituted or unsubstituted heteroaryl terminated with a functional group. The above structures act as reactive protective groups that can be reacted with, for example, a side chain of an amino acid of a targeting agent, such as an antibody, to thereby link the targeting agent to the linker-drug moiety.

Targeting Agents

The linker arms and cytotoxins of the invention can be linked to targeting agents that selectively deliver a payload to a cell, organ or region of the body. Exemplary targeting agents such as antibodies (e.g., chimeric, humanized and human), ligands for receptors, lectins, saccharides, antibodies, and the like are recognized in the art and are useful without limitation in practicing the present invention. Other targeting agents include a class of compounds that do not include specific molecular recognition motifs include macromolecules such as poly(ethylene glycol), polysaccharide, polyamino acids and the like, which add molecular mass to the cytotoxin. The additional molecular mass affects the pharmacokinetics of the cytotoxin, e.g., serum half-life.

In an exemplary embodiment, the invention provides a cytotoxin, linker or cytotoxin-linker conjugate with a targeting agent that is a biomolecule, e.g, an antibody, receptor, peptide, lectin, saccharide, nucleic acid or a combination thereof. Routes to exemplary conjugates of the invention are set forth in the Schemes above.

Biomolecules useful in practicing the present invention can be derived from any source. The biomolecules can be isolated from natural sources or can be produced by synthetic methods. Proteins can be natural proteins or mutated proteins. Mutations can be effected by chemical mutagenesis, site-directed mutagenesis or other means of inducing mutations known to those of skill in the art. Proteins useful in practicing the instant invention include, for example, enzymes, antigens, antibodies and receptors. Antibodies can be either polyclonal or monoclonal, but most preferably are monoclonal. Peptides and nucleic acids can be isolated from natural sources or can be wholly or partially synthetic in origin.

In a preferred embodiment, the targeting agent is an antibody, or antibody fragment, that is selected based on its specificity for an antigen expressed on a target cell, or at a target site, of interest. A wide variety of tumor-specific or other disease-specific antigens have been identified and antibodies to those antigens have been used or proposed for use in the treatment of such tumors or other diseases. The antibodies that are known in the art can be used in the conjugates of the invention, in particular for the treatment of the disease with which the target antigen is associated. Non-limiting examples of target antigens (and their associated diseases) to which an antibody-linker-drug conjugate of the invention can be targeted include: Her2 (breast cancer), CD20 (lymphomas), EGFR (solid tumors), CD22 (lymphomas, including

non-Hodgkin's lymphoma), CD52 (chronic lymphocytic leukemia), CD33 (acute myelogenous leukemia), CD4 (lymphomas, autoimmune diseases, including rheumatoid arthritis), CD30 (lymphomas, including non-Hodgkin's lymphoma), Muc18 (melanoma), integrins (solid tumors), PSMA (prostate cancer, benign prostatic hyperplasia), CEA (colorectal cancer), CD11a (psoriasis), CD80 (psoriasis), CD23 (asthma), CD40L (immune thromobcytopenic purpura), CTLA4 (T cell lymphomas) and BLys (autoimmune diseases, including systemic lupus erythematosus).

In those embodiments wherein the recognition moiety is a protein or antibody, the protein can be tethered to a surface or a self assembled monolayer (SAM) component or connected through a spacer arm by any reactive peptide residue available on the surface of the protein. In preferred embodiments, the reactive groups are amines or carboxylates. In particularly preferred embodiments, the reactive groups are the ε-amine groups of lysine residues. Furthermore, these molecules can be adsorbed onto the surface of the substrate or SAM by non-specific interactions (*e.g.*, chemisorption, physisorption).

Recognition moieties which are antibodies can be used to recognize analytes which are proteins, peptides, nucleic acids, saccharides or small molecules such as drugs, herbicides, pesticides, industrial chemicals and agents of war. Methods of raising antibodies for specific molecules are well-known to those of skill in the art. *See*, United States Patents No. 5/147,786, issued to Feng *et al.* on September 15, 1992; No. 5/334,528, issued to Stanker *et al.* on August 2, 1994; No. 5/686,237, issued to Al-Bayati, M.A.S. on November 11, 1997; and No. 5/573,922, issued to Hoess *et al.* on November 12, 1996. Methods for attaching antibodies to surfaces are also art-known. *See*, Delamarche *et al. Langmuir* **12:**1944-1946 (1996).

Targeting agents can be attached to the linkers of the invention by any available reactive group. For example, peptides can be attached through an amine, carboxyl, sulfhydryl, or hydroxyl group. Such a group can reside at a peptide terminus or at a site internal to the peptide chain. Nucleic acids can be attached through a reactive group on a base (*e.g.*, exocyclic amine) or an available hydroxyl group on a sugar moiety (*e.g.*, 3'- or 5'-hydroxyl). The peptide and nucleic acid chains can be further derivatized at one or more sites to allow for the attachment of appropriate reactive groups onto the chain. *See*, Chrisey *et al. Nucleic Acids Res.* **24:**3031-3039 (1996).

When the peptide or nucleic acid is a fully or partially synthetic molecule, a reactive group or masked reactive group can be incorporated during the process of the synthesis. Many derivatized monomers appropriate for reactive group incorporation in both peptides and nucleic acids are know to those of skill in the art. *See*, for example, THE PEPTIDES: ANALYSIS, SYNTHESIS, BIOLOGY, Vol. 2: "Special Methods in Peptide Synthesis," Gross, E. and Melenhofer, J., Eds., Academic Press, New York (1980). Many useful monomers are commercially available (Bachem, Sigma, *etc.*). This masked group can then be unmasked following the synthesis, at which time it becomes available for reaction with a component of a compound of the invention.

Exemplary nucleic acid targeting agents include aptamers, antisense compounds, and nucleic acids that form triple helices. Typically, a hydroxyl group of a sugar residue, an amino group from a base residue, or a phosphate oxygen of the nucleotide is utilized as the needed chemical functionality to couple the nucleotide-based targeting agent to the cytotoxin. However, one of skill in the art will readily appreciate that other "non-natural" reactive functionalities can be appended to a nucleic acid by conventional techniques. For example, the hydroxyl group of the sugar residue can be converted to a mercapto or amino group using techniques well known in the art.

Aptamers (or nucleic acid antibody) are single- or double-stranded DNA or single-stranded RNA molecules that bind specific molecular targets. Generally, aptamers function by inhibiting the actions of the molecular target, *e.g.*, proteins, by binding to the pool of the target circulating in the blood. Aptamers possess chemical functionality and thus, can covalently bond to cytotoxins, as described herein.

Although a wide variety of molecular targets are capable of forming non-covalent but specific associations with aptamers, including small molecules drugs, metabolites, cofactors, toxins, saccharide-based drugs, nucleotide-based drugs, glycoproteins, and the like, generally the molecular target will comprise a protein or peptide, including serum proteins, kinins, eicosanoids, cell surface molecules, and the like. Examples of aptamers include Gilead's antithrombin inhibitor GS 522 and its derivatives (Gilead Science, Foster City, Calif.). *See also*, Macaya *et al. Proc. Natl. Acad. Sci. USA* **90**:3745-9 (1993); Bock *et al. Nature (London)* **355**:564-566 (1992) and Wang *et al. Biochem.* **32**:1899-904 (1993).

Aptamers specific for a given biomolecule can be identified using techniques known in the art. *See, e.g.*, Toole *et al.* (1992) PCT Publication No. WO 92/14843; Tuerk and Gold (1991) PCT Publication No. WO 91/19813; Weintraub and Hutchinson (1992) PCT Publication No. 92/05285; and Ellington and Szostak, *Nature* **346**:818 (1990). Briefly, these techniques typically involve the complexation of the molecular target with a random mixture of oligonucleotides. The aptamer-molecular target complex is separated from the uncomplexed oligonucleotides. The aptamer is recovered from the separated complex and amplified. This cycle is repeated to identify those aptamer sequences with the highest affinity for the molecular target.

For diseases that result from the inappropriate expression of genes, specific prevention or reduction of the expression of such genes represents an ideal therapy. In principle, production of a particular gene product may be inhibited, reduced or shut off by hybridization of a single-stranded deoxynucleotide or ribodeoxynucleotide complementary to an accessible sequence in the mRNA, or a sequence within the transcript that is essential for pre-mRNA processing, or to a sequence within the gene itself. This paradigm for genetic control is often referred to as antisense or antigene inhibition. Additional efficacy is imparted by the conjugation to the nucleic acid of an alkylating agent, such as those of the present invention.

Antisense compounds are nucleic acids designed to bind and disable or prevent the production of the mRNA responsible for generating a particular protein. Antisense compounds include antisense RNA or DNA, single or double stranded, oligonucleotides, or their analogs, which can hybridize specifically to individual mRNA species and prevent transcription and/or RNA processing of the mRNA species and/or translation of the encoded polypeptide and thereby effect a reduction in the amount of the respective encoded polypeptide. Ching *et al. Proc. Natl. Acad. Sci. U.S.A.* **86**:10006-10010 (1989); Broder *et al. Ann. Int. Med.* **113**:604-618 (1990); Loreau *et al. FEBS Letters* **274**:53-56 (1990); Holcenberg *et al.* WO91/11535; WO91/09865; WO91/04753; WO90/13641; WO 91/13080, WO 91/06629, and EP 386563). Due to their exquisite target sensitivity and selectivity, antisense oligonucleotides are useful for delivering therapeutic agents, such as the cytotoxins of the invention to a desired molecular target.

Others have reported that nucleic acids can bind to duplex DNA via triple helix formation and inhibit transcription and/or DNA synthesis. Triple helix compounds (also referred

to as triple strand drugs) are oligonucleotides that bind to sequences of double-stranded DNA and are intended to inhibit selectively the transcription of disease-causing genes, such as viral genes, *e.g.*, HIV and herpes simplex virus, and oncogenes, *i.e.*, they stop protein production at the cell nucleus. These drugs bind directly to the double stranded DNA in the cell's genome to form a triple helix and prevent the cell from making a target protein. *See, e.g.*, PCT publications Nos. WO 92/10590, WO 92/09705, WO91/06626, and U.S. Pat. No. 5,176,996. Thus, the cytotoxins of the present invention are also conjugated to nucleic acid sequences that form triple helices.

The site specificity of nucleic acids (*e.g.*, antisense compounds and triple helix drugs) is not significantly affected by modification of the phosphodiester linkage or by chemical modification of the oligonucleotide terminus. Consequently, these nucleic acids can be chemically modified; enhancing the overall binding stability, increasing the stability with respect to chemical degradation, increasing the rate at which the oligonucleotides are transported into cells, and conferring chemical reactivity to the molecules. The general approach to constructing various nucleic acids useful in antisense therapy has been reviewed by van der Krol *et al.*, *Biotechniques* **6**:958-976 (1988) and Stein *et al. Cancer Res.* **48**:2659-2668 (1988). Therefore, in an exemplary embodiment, the cytotoxins of the invention are conjugated to a nucleic acid by modification of the phosphodiester linkage.

Moreover, aptamers, antisense compounds and triple helix drugs bearing cytotoxins of the invention can also can include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridization to or association with the relevant target sequence is retained as a functional property of the oligonucleotide. For example, some embodiments will employ phosphorothioate analogs which are more resistant to degradation by nucleases than their naturally occurring phosphate diester counterparts and are thus expected to have a higher persistence in vivo and greater potency (*see, e.g.*, Campbell *et al., J. Biochem. Biophys. Methods* **20**:259-267(1990)). Phosphoramidate derivatives of oligonucleotides also are known to bind to complementary polynucleotides and have the additional capability of accommodating covalently attached ligand species and will be amenable to the methods of the present invention. *See*, for example, Froehler *et al., Nucleic Acids Res.* **16**(11):4831 (1988).

In some embodiments the aptamers, antisense compounds and triple helix drugs will comprise O-methylribonucleotides (EP Publication No. 360609). Chimeric oligonucleotides

may also be used (Dagle *et al., Nucleic Acids Res.* **18**: 4751 (1990)). For some applications, antisense oligonucleotides and triple helix may comprise polyamide nucleic acids (Nielsen *et al., Science* **254**: 1497 (1991) and PCT publication No. WO 90/15065) or other cationic derivatives (Letsinger *et al., J. Am. Chem. Soc.* **110**: 4470-4471 (1988)). Other applications may utilize oligonucleotides wherein one or more of the phosphodiester linkages has been substituted with an isosteric group, such as a 2-4 atom long internucleoside linkage as described in PCT publication Nos. WO 92/05186 and 91/06556, or a formacetal group (Matteucci *et al., J. Am. Chem. Soc.* **113**: 7767-7768 (1991)) or an amide group (Nielsen *et al., Science* **254**: 1497-1500 (1991)).

In addition, nucleotide analogs, for example wherein the sugar or base is chemically modified, can be employed in the present invention. "Analogous" forms of purines and pyrimidines are those generally known in the art, many of which are used as chemotherapeutic agents. An exemplary but not exhaustive list includes 4-acetylcytosine, 5-(carboxyhydroxylmethyl) uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N^6 -isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N^6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil, β-D-mannosylqueosine, 5'methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N^6 -isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), wybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid (v), pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine. In addition, the conventional bases by halogenated bases. Furthermore, the 2'-furanose position on the base can have a non-charged bulky group substitution. Examples of non-charged bulky groups include branched alkyls, sugars and branched sugars.

Terminal modification also provides a useful procedure to conjugate the cytotoxins to the nucleic acid, modify cell type specificity, pharmacokinetics, nuclear permeability, and absolute cell uptake rate for oligonucleotide pharmaceutical agents. For example, an array of substitutions at the 5' and 3' ends to include reactive groups are known, which allow covalent attachment of the cytotoxins. See, *e.g.*, OLIGODEOXYNUCLEOTIDES:

ANTISENSE INHIBITORS OF GENE EXPRESSION, (1989) Cohen, Ed., CRC Press; PROSPECTS FOR ANTISENSE NUCLEIC ACID THERAPEUTICS FOR CANCER AND AIDS, (1991), Wickstrom, Ed., Wiley-Liss; GENE REGULATION: BIOLOGY OF ANTISENSE RNA AND DNA, (1992) Erickson and Izant, Eds., Raven Press; and ANTISENSE RNA AND DNA, (1992), Murray, Ed., Wiley-Liss. For general methods relating to antisense compounds, *see*, ANTISENSE RNA AND DNA, (1988), D. A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.).

Detectable Labels

The particular label or detectable group used in conjunction with the compounds and methods of the invention is generally not a critical aspect of the invention, as long as it does not significantly interfere with the activity or utility of the compound of the invention. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (e.g., DYNABEADS^{TM}), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, Texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads (*e.g.*, polystyrene, polypropylene, latex, etc.).

The label may be coupled directly or indirectly to a compound of the invention according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

When the compound of the invention is conjugated to a detectable label, the label is preferably a member selected from the group consisting of radioactive isotopes, fluorescent agents, fluorescent agent precursors, chromophores, enzymes and combinations thereof. Methods for conjugating various groups to antibodies are well known in the art. For example, a

detectable label that is frequently conjugated to an antibody is an enzyme, such as horseradish peroxidase, alkaline phosphatase, β-galactosidase, and glucose oxidase.

Non-radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to a component of the conjugate. The ligand then binds to another molecules (*e.g.*, streptavidin) molecule, which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound.

Components of the conjugates of the invention can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidotases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, *etc.* Chemiluminescent compounds include luciferin, and 2,3-dihydrophthalazinediones, *e.g.*, luminol. For a review of various labeling or signal producing systems that may be used, *see*, U.S. Patent No. 4,391,904.

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorochrome with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Fluorescent labels are presently preferred as they have the advantage of requiring few precautions in handling, and being amenable to high-throughput visualization techniques (optical analysis including digitization of the image for analysis in an integrated system comprising a computer). Preferred labels are typically characterized by one or more of the

following: high sensitivity, high stability, low background, low environmental sensitivity and high specificity in labeling. Many fluorescent labels are commercially available from the SIGMA chemical company (Saint Louis, MO), Molecular Probes (Eugene, OR), R&D systems (Minneapolis, MN), Pharmacia LKB Biotechnology (Piscataway, NJ), CLONTECH Laboratories, Inc. (Palo Alto, CA), Chem Genes Corp., Aldrich Chemical Company (Milwaukee, WI), Glen Research, Inc., GIBCO BRL Life Technologies, Inc. (Gaithersburg, MD), Fluka Chemica- Biochemika Analytika (Fluka Chemie AG, Buchs, Switzerland), and Applied Biosystems (Foster City, CA), as well as many other commercial sources known to one of skill. Furthermore, those of skill in the art will recognize how to select an appropriate fluorophore for a particular application and, if it not readily available commercially, will be able to synthesize the necessary fluorophore de novo or synthetically modify commercially available fluorescent compounds to arrive at the desired fluorescent label.

In addition to small molecule fluorophores, naturally occurring fluorescent proteins and engineered analogues of such proteins are useful in the present invention. Such proteins include, for example, green fluorescent proteins of cnidarians (Ward *et al., Photochem. Photobiol.* **35**:803-808 (1982); Levine *et al., Comp. Biochem. Physiol.*, **72B**:77-85 (1982)), yellow fluorescent protein from Vibrio *fischeri* strain (Baldwin *et al., Biochemistry* **29**:5509-15 (1990)), Peridinin-chlorophyll from the dinoflagellate Symbiodinium sp. (Morris *et al., Plant Molecular Biology* **24**:673:77 (1994)), phycobiliproteins from marine cyanobacteria, such as Synechococcus, *e.g.*, phycoerythrin and phycocyanin (Wilbanks *et al., J. Biol. Chem.* **268**:1226-35 (1993)), and the like.

Generally, prior to forming the linkage between the cytotoxin and the targeting (or other) agent, and optionally, the spacer group, at least one of the chemical functionalities will be activated. One skilled in the art will appreciate that a variety of chemical functionalities, including hydroxy, amino, and carboxy groups, can be activated using a variety of standard methods and conditions. For example, a hydroxyl group of the cytotoxin or targeting agent can be activated through treatment with phosgene to form the corresponding chloroformate, or p-nitrophenylchloroformate to form the corresponding carbonate.

In an exemplary embodiment, the invention makes use of a targeting agent that includes a carboxyl functionality. Carboxyl groups may be activated by, for example, conversion to the corresponding acyl halide or active ester. This reaction may be performed

under a variety of conditions as illustrated in March, supra pp. 388-89. In an exemplary embodiment, the acyl halide is prepared through the reaction of the carboxyl-containing group with oxalyl chloride. The activated agent is reacted with a cytotoxin or cytotoxin-linker arm combination to form a conjugate of the invention. Those of skill in the art will appreciate that the use of carboxyl-containing targeting agents is merely illustrative, and that agents having many other functional groups can be conjugated to the linkers of the invention.

Reactive Functional Groups

For clarity of illustration the succeeding discussion focuses on the conjugation of a cytotoxin of the invention to a targeting agent. The focus exemplifies one embodiment of the invention from which, others are readily inferred by one of skill in the art. No limitation of the invention is implied, by focusing the discussion on a single embodiment.

Exemplary compounds of the invention bear a reactive functional group, which is generally located on a substituted or unsubstituted alkyl or heteroalkyl chain, allowing their facile attachment to another species. A convenient location for the reactive group is the terminal position of the chain.

Reactive groups and classes of reactions useful in practicing the present invention are generally those that are well known in the art of bioconjugate chemistry. The reactive functional group may be protected or unprotected, and the protected nature of the group may be changed by methods known in the art of organic synthesis. Currently favored classes of reactions available with reactive cytotoxin analogues are those which proceed under relatively mild conditions. These include, but are not limited to nucleophilic substitutions (*e.g.*, reactions of amines and alcohols with acyl halides, active esters), electrophilic substitutions (*e.g.*, enamine reactions) and additions to carbon-carbon and carbon-heteroatom multiple bonds (*e.g.*, Michael reaction, Diels-Alder addition). These and other useful reactions are discussed in, for example, March, ADVANCED ORGANIC CHEMISTRY, 3rd Ed., John Wiley & Sons, New York, 1985; Hermanson, BIOCONJUGATE TECHNIQUES, Academic Press, San Diego, 1996; and Feeney *et al.*, MODIFICATION OF PROTEINS; Advances in Chemistry Series, Vol. 198, American Chemical Society, Washington, D.C., 1982.

Exemplary reaction types include the reaction of carboxyl groups and various derivatives thereof including, but not limited to, N-hydroxysuccinimide esters, N-

hydroxybenztriazole esters, acid halides, acyl imidazoles, thioesters, p-nitrophenyl esters, alkyl, alkenyl, alkynyl and aromatic esters. Hydroxyl groups can be converted to esters, ethers, aldehydes, *etc.* Haloalkyl groups are converted to new species by reaction with, for example, an amine, a carboxylate anion, thiol anion, carbanion, or an alkoxide ion. Dienophile (e.g., maleimide) groups participate in Diels-Alder. Aldehyde or ketone groups can be converted to imines, hydrazones, semicarbazones or oximes, or via such mechanisms as Grignard addition or alkyllithium addition. Sulfonyl halides react readily with amines, for example, to form sulfonamides. Amine or sulfhydryl groups are, for example, acylated, alkylated or oxidized. Alkenes, can be converted to an array of new species using cycloadditions, acylation, Michael addition, *etc.* Epoxides react readily with amines and hydroxyl compounds.

One skilled in the art will readily appreciate that many of these linkages may be produced in a variety of ways and using a variety of conditions. For the preparation of esters, *see, e.g.*, March *supra* at 1157; for thioesters, *see*, March, *supra* at 362-363, 491, 720-722, 829, 941, and 1172; for carbonates, *see*, March, *supra* at 346-347; for carbamates, *see*, March, *supra* at 1156-57; for amides, *see*, March *supra* at 1152; for ureas and thioureas, *see*, March *supra* at 1174; for acetals and ketals, *see*, Greene *et al. supra* 178-210 and March *supra* at 1146; for acyloxyalkyl derivatives, *see*, PRODRUGS: TOPICAL AND OCULAR DRUG DELIVERY, K. B. Sloan, ed., Marcel Dekker, Inc., New York, 1992; for enol esters, *see*, March supra at 1160; for N-sulfonylimidates, *see*, Bundgaard *et al.*, *J. Med. Chem.*, **31**:2066 (1988); for anhydrides, *see*, March *supra* at 355-56, 636-37, 990-91, and 1154; for N-acylamides, *see*, March *supra* at 379; for N-Mannich bases, *see*, March *supra* at 800-02, and 828; for hydroxymethyl ketone esters, *see*, Petracek *et al. Annals NY Acad. Sci.*, **507**:353-54 (1987); for disulfides, *see*, March *supra* at 1160; and for phosphonate esters and phosphonamidates.

The reactive functional groups can be unprotected and chosen such that they do not participate in, or interfere with, the reactions. Alternatively, a reactive functional group can be protected from participating in the reaction by the presence of a protecting group. Those of skill in the art will understand how to protect a particular functional group from interfering with a chosen set of reaction conditions. For examples of useful protecting groups, *See* Greene *et al.*, PROTECTIVE GROUPS IN ORGANIC SYNTHESIS, John Wiley & Sons, New York, 1991.

Typically, the targeting agent is linked covalently to a cytotoxin using standard chemical techniques through their respective chemical functionalities. Optionally, the linker or

agent is coupled to the agent through one or more spacer groups. The spacer groups can be equivalent or different when used in combination.

Generally, prior to forming the linkage between the cytotoxin and the reactive functional group, and optionally, the spacer group, at least one of the chemical functionalities will be activated. One skilled in the art will appreciate that a variety of chemical functionalities, including hydroxy, amino, and carboxy groups, can be activated using a variety of standard methods and conditions. In an exemplary embodiment, the invention comprises a carboxyl functionality as a reactive functional group. Carboxyl groups may be activated as described hereinabove.

PHARMACEUTICAL FORMULATIONS AND ADMINISTRATION

In another preferred embodiment, the present invention provides a pharmaceutical formulation comprising a compound of the invention and a pharmaceutically acceptable carrier.

The compounds described herein including pharmaceutically acceptable carriers such as addition salts or hydrates thereof, can be delivered to a patient using a wide variety of routes or modes of administration. Suitable routes of administration include, but are not limited to, inhalation, transdermal, oral, rectal, transmucosal, intestinal and parenteral administration, including intramuscular, subcutaneous and intravenous injections. Preferably, the conjugates of the invention comprising an antibody or antibody fragment as the targeting moiety are administered parenterally, more preferably intravenously.

As used herein, the terms "administering" or "administration" are intended to encompass all means for directly and indirectly delivering a compound to its intended site of action.

The compounds described herein, or pharmaceutically acceptable salts and/or hydrates thereof, may be administered singly, in combination with other compounds of the invention, and/or in cocktails combined with other therapeutic agents. Of course, the choice of therapeutic agents that can be co-administered with the compounds of the invention will depend, in part, on the condition being treated.

For example, when administered to patients suffering from a disease state caused by an organism that relies on an autoinducer, the compounds of the invention can be administered in cocktails containing agents used to treat the pain, infection and other symptoms

and side effects commonly associated with the disease. Such agents include, *e.g.*, analgesics, antibiotics, *etc.*

When administered to a patient undergoing cancer treatment, the compounds may be administered in cocktails containing anti-cancer agents and/or supplementary potentiating agents. The compounds may also be administered in cocktails containing agents that treat the side-effects of radiation therapy, such as anti-emetics, radiation protectants, *etc.*

Supplementary potentiating agents that can be co-administered with the compounds of the invention include, *e.g.*, tricyclic anti-depressant drugs (*e.g.*, imipramine, desipramine, amitriptyline, clomipramine, trimipramine, doxepin, nortriptyline, protriptyline, amoxapine and maprotiline); non-tricyclic and anti-depressant drugs (*e.g.*, sertraline, trazodone and citalopram); Ca^{+2} antagonists (*e.g.*, verapamil, nifedipine, nitrendipine and caroverine); amphotericin; triparanol analogues (*e.g.*, tamoxifen); antiarrhythmic drugs (*e.g.*, quinidine); antihypertensive drugs (*e.g.*, reserpine); thiol depleters (*e.g.*, buthionine and sulfoximine); and calcium leucovorin.

The active compound(s) of the invention are administered *per se* or in the form of a pharmaceutical composition wherein the active compound(s) is in admixture with one or more pharmaceutically acceptable carriers, excipients or diluents. Pharmaceutical compositions for use in accordance with the present invention are typically formulated in a conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active compounds into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compound(s) with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a patient to be treated. Pharmaceutical preparations for oral use can be obtained solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired. to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations

such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxyniethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used, which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical preparations, which can be used orally, include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with filler such as lactose, binders such as starches, and/or lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for such administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by inhalation, the compounds for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from pressurized packs or a nebulizer, with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurized aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The compounds may be formulated for parenteral administration by injection, *e.g.*, by bolus injection or continuous infusion. Injection is a preferred method of administration for the compositions of the current invention. Formulations for injection may be presented in

unit dosage form, e.g., in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Pharmaceutical formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers or agents, which increase the solubility of the compounds to allow for the preparation of highly, concentrated solutions. For injection, the agents of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks's solution, Ringer's solution, or physiological saline buffer.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

The compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation or transcutaneous delivery (*e.g.*, subcutaneously or intramuscularly), intramuscular injection or a transdermal patch. Thus, for example, the compounds may be formulated with suitable polymeric or hydrophobic materials (*e.g.*, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt.

The pharmaceutical compositions also may comprise suitable solid or gel phase carriers or excipients. Examples of such carriers or excipients include but are not limited to calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

A preferred pharmaceutical composition is a composition formulated for injection such as intravenous injection and includes about 0.01% to about 100% by weight of the drug-ligand conjugate, based upon 100% weight of total pharmaceutical composition. The drug-ligand conjugate may be an antibody-cytotoxin conjugate where the antibody has been selected to target a particular cancer.

LIBRARIES

Also within the scope of the present invention are libraries of the cytotoxin, cytotoxin-linker and agent-linker conjugates of the cytotoxins and linkers of the invention. Exemplary libraries include at least 10 compounds, more preferably at least 100 compound, even more preferably at least 1000 compounds and still more preferably at least 100,000 compounds. The libraries in a form that is readily queried for a particular property, e.g., cytotoxicity, cleavage of a linker by an enzyme, or other cleavage reagent. Exemplary forms include chip formats, microarrays, and the like.

Parallel, or combinatorial, synthesis has as its primary objective the generation of a library of diverse molecules which all share a common feature, referred to throughout this description as a scaffold. By substituting different moieties at each of the variable parts of the scaffold molecule, the amount of space explorable in a library grows. Theories and modern medicinal chemistry advocate the concept of occupied space as a key factor in determining the efficacy of a given compound against a given biological target. By creating a diverse library of molecules, which explores a large percentage of the targeted space, the odds of developing a highly efficacious lead compound increase dramatically.

Parallel synthesis is generally conducted on a solid phase support, such as a polymeric resin. The scaffold, or other suitable intermediate is cleavably tethered to the resin by a chemical linker. Reactions are carried out to modify the scaffold while tethered to the particle. Variations in reagents and/or reaction conditions produce the structural diversity, which is the hallmark of each library.

Parallel synthesis of "small" molecules (non-oligomers with a molecular weight of 200-1000) was rarely attempted prior to 1990. *See*, for example, Camps. *et al.*, *Annaks de Quimica,* **70**: 848 (1990). Recently, Ellmann disclosed the solid phase-supported parallel (also referred to as "combinatorial") synthesis of eleven benzodiazepine analogs along with some

prostaglandins and beta-turn mimetics. These disclosures are exemplified in U.S. Pat. No. 5,288,514. Another relevant disclosure of parallel synthesis of small molecules may be found in U.S. Pat. No. 5,324,483. This patent discloses the parallel synthesis of between 4 and 40 compounds in each of sixteen different scaffolds. Chen *et al.* have also applied organic synthetic strategies to develop non-peptide libraries synthesized using multi-step processes on a polymer support. (Chen *et al., J. Am. Chem. Soc.*, **116**: 2661-2662 (1994)).

Once a library of unique compounds is prepared, the preparation of a library of immunoconjugates, or antibodies can be prepared using the library of autoinducers as a starting point and using the methods described herein.

KITS

In another aspect, the present invention provides kits containing one or more of the compounds or compositions of the invention and directions for using the compound or composition. In an exemplary embodiment, the invention provides a kit for conjugating a linker arm of the invention to another molecule. The kit includes the linker, and directions for attaching the linker to a particular functional group. The kit may also include one or more of a cytotoxic drug, a targeting agent, a detectable label, pharmaceutical salts or buffers. The kit may also include a container and optionally one or more vial, test tube, flask, bottle, or syringe. Other formats for kits will be apparent to those of skill in the art and are within the scope of the present invention.

PURIFICATION

In another exemplary embodiment, the present invention provides a method for isolating a molecular target for a ligand-cytotoxin of the invention, which binds to the ligand X^4. The method preferably comprises, contacting a cellular preparation that includes the target with an immobilized compound, thereby forming a complex between the receptor and the immobilized compound.

The cytotoxin of the invention can be immobilized on an affinity support by any art-recognized means. Alternatively, the cytotoxin can be immobilized using one or more of the linkers of the invention.

In yet another exemplary embodiment, the invention provides an affinity purification matrix that includes a linker of the invention.

The method of the invention for isolating a target will typically utilize one or more affinity chromatography techniques. Affinity chromatography enables the efficient isolation of species such as biological molecules or biopolymers by utilizing their recognition sites for certain supported chemical structures with a high degree of selectivity. The literature is replete with articles, monographs, and books on the subject of affinity chromatography, including such topics as affinity chromatography supports, crosslinking members, ligands and their preparation and use. A sampling of those references includes: Ostrove, *Methods Enzymol.* **182**: 357-71 (1990); Ferment, *Bioeng.* **70**: 199-209 (1990). Huang *et al.*, *J. Chromatogr.* **492**: 431-69 (1989); "Purification of enzymes by heparin-Sepharose affinity chromatography," *J. Chromatogr.*, **184**: 335-45 (1980); Farooqi, *Enzyme Eng.*, **4**: 441-2 (1978); Nishikawa, *Chem. Technol.*, **5(9)**: 564-71 (1975); Guilford *et al.*, in, PRACT. HIGH PERFORM. LIQ. CHROMATOGR., Simpson (ed.), 193-206 (1976); Nishikawa, *Proc. Int. Workshop Technol. Protein Sep. Improv. Blood Plasma Fractionation*, Sandberg (ed.), 422-35; (1977) "Affinity chromatography of enzymes," *Affinity Chromatogr., Proc. Int. Symp.* 25-38, (1977) (Pub. 1978); and AFFINITY CHROMATOGRAPHY: A PRACTICAL APPROACH, Dean *et al.* (ed.), IRL Press Limited, Oxford, England (1985). Those of skill in the art have ample guidance in developing particular affinity chromatographic methods utilizing the materials of the invention.

In the present method, affinity chromatographic media of varying chemical structures can be used as supports. For example, agarose gels and cross-linked agarose gels are useful as support materials, because their hydrophilicity makes them relatively free of nonspecific binding. Other useful supports include, for example, controlled-pore glass (CPG) beads, cellulose particles, polyacrylamide gel beads and Sephadex™ gel beads made from dextran and epichlorohydrin.

DRUG-LIGAND CONJUGATE METHODS OF USE

In addition to the compositions and constructs described above, the present invention also provides a number of methods that can be practiced utilizing the compounds and conjugates of the invention. Methods for using the drug-ligand conjugate of the current invention include: killing or inhibiting the growth or replication of a tumor cell or cancer cell,

treating cancer, treating a pre-cancerous condition, killing or inhibiting the growth or replication of a cell that expresses an auto-immune antibody, treating an autoimmune disease, treating an infectious disease, preventing the multiplication of a tumor cell or cancer cell, preventing cancer, preventing the multiplication of a cell that expresses an auto-immune antibody, preventing an autoimmune disease, and preventing an infectious disease. These methods of use comprise administering to an animal such as a mammal or a human in need thereof an effective amount of a drug-ligand conjugate. Preferred ligands for many of the methods of use described herein include antibodies and antibody fragments which target the particular tumor cell, cancer cell, or other target area.

The drug-ligand complex of the current invention is useful for treating cancer, autoimmune disease and infectious disease in an animal. Compositions and methods for treating tumors by providing a subject the composition in a pharmaceutically acceptable manner, with a pharmaceutically effective amount of a composition of the present invention are provided.

The current invention is particularly useful for the treatment of cancer and for the inhibition of the multiplication of a tumor cell or cancer cell in an animal. Cancer, or a precancerous condition, includes, but is not limited to, a tumor, metastasis, or any disease or disorder characterized by uncontrolled cell growth, can be treated or prevented by administration the drug-ligand complex of the current invention. The complex delivers the drug a tumor cell or cancer cell. In one embodiment, the ligand specifically binds to or associates with a cancer-cell or a tumor-cell-associated antigen. Because of its close proximity to the ligand, the drug can be taken up inside a tumor cell or cancer cell through, for example, receptor-mediated endocytosis. The antigen can be attached to a tumor cell or cancer cell or can be an extracellular matrix protein associated with the tumor cell or cancer cell. Once inside the cell, the linker is hydrolytically cleaved by a tumor-cell or cancer-cell-associated proteases, thereby releasing the drug. The released drug is then free to diffuse and induce cytotoxic activities. In an alternative embodiment, the drug is cleaved from the drug-ligand complex outside the tumor cell or cancer cell, and the drug subsequently penetrates the cell.

The ligand may bind to, for example, a tumor cell or cancer cell, a tumor cell or cancer cell antigen which is on the surface of the tumor cell or cancer cell, or a tumor cell or cancer cell antigen which is an extracellular matrix protein associated with the tumor cell or cancer cell. The ligand can be designed specifically for a particular tumor cell or cancer cell

type. Therefore, the type of tumors or cancers that can be effectively treated can be altered by the choice of ligand.

Representative examples of precancerous conditions that may be targeted by the drug-ligand conjugate, include, but are not limited to: metaplasia, hyperplasia, dysplasia, colorectal polyps, actinic ketatosis, actinic cheilitis, human papillomaviruses, leukoplakia, lychen planus and Bowen's disease.

Representative examples of cancers or tumors that may be targeted by the drug-ligand conjugate include, but are not limited to: lung cancer, colon cancer, prostate cancer, lymphoma, melanoma, breast cancer, ovarian cancer, testicular cancer, CNS cancer, renal cancer, kidney cancer, pancreatic cancer, stomach cancer, oral cancer, nasal cancer, cervical cancer and leukemias. It will be readily apparent to the ordinarily skilled artisan that the particular targeting ligand used in the conjugate can be chosen such that it targets the drug to the tumor tissue to be treated with the drug (i.e., a targeting agent specific for a tumor-specific antigen is chosen). Examples of such targeting ligands are well known in the art, non-limiting examples of which include anti-Her2 for treatment of breast cancer, anti-CD20 for treatment of lymphoma, anti-PSMA for treatment of prostate cancer and anti-CD30 for treatment of lymphomas, including non-Hodgkin's lymphoma.

In an embodiment, the present invention provides a method of killing a cell. The method includes administering to the cell an amount of a compound of the invention sufficient to kill said cell. In an exemplary embodiment, the compound is administered to a subject bearing the cell. In a further exemplary embodiment, the administration serves to retard or stop the growth of a tumor that includes the cell (*e.g.*, the cell can be a tumor cell). For the administration to retard the growth, the rate of growth of the cell should be at least 10% less than the rate of growth before administration. Preferably, the rate of growth will be retarded at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or completely stopped.

Effective Dosages

Pharmaceutical compositions suitable for use with the present invention include compositions wherein the active ingredient is contained in a therapeutically effective amount, *i.e.*, in an amount effective to achieve its intended purpose. The actual amount effective for a particular application will depend, *inter alia,* on the condition being treated. Determination of

an effective amount is well within the capabilities of those skilled in the art, especially in light of the detailed disclosure herein.

For any compound described herein, the therapeutically effective amount can be initially determined from cell culture assays. Target plasma concentrations will be those concentrations of active compound(s) that are capable of inhibition cell growth or division. In preferred embodiments, the cellular activity is at least 25% inhibited. Target plasma concentrations of active compound(s) that are capable of inducing at least about 50%, 75%, or even 90% or higher inhibition of cellular activity are presently preferred. The percentage of inhibition of cellular activity in the patient can be monitored to assess the appropriateness of the plasma drug concentration achieved, and the dosage can be adjusted upwards or downwards to achieve the desired percentage of inhibition.

As is well known in the art, therapeutically effective amounts for use in humans can also be determined from animal models. For example, a dose for humans can be formulated to achieve a circulating concentration that has been found to be effective in animals. The dosage in humans can be adjusted by monitoring cellular inhibition and adjusting the dosage upwards or downwards, as described above.

A therapeutically effective dose can also be determined from human data for compounds which are known to exhibit similar pharmacological activities. The applied dose can be adjusted based on the relative bioavailability and potency of the administered compound as compared with the known compound.

Adjusting the dose to achieve maximal efficacy in humans based on the methods described above and other methods as are well-known in the art is well within the capabilities of the ordinarily skilled artisan.

In the case of local administration, the systemic circulating concentration of administered compound will not be of particular importance. In such instances, the compound is administered so as to achieve a concentration at the local area effective to achieve the intended result.

For use in the prophylaxis and/or treatment of diseases related to abnormal cellular proliferation, a circulating concentration of administered compound of about 0.001 μM to 20 μM is preferred, with about 0.01 μM to 5 μM being preferred.

Patient doses for oral administration of the compounds described herein, typically range from about 1 mg/day to about 10,000 mg/day, more typically from about 10 mg/day to about 1,000 mg/day, and most typically from about 50 mg/day to about 500 mg/day. Stated in terms of patient body weight, typical dosages range from about 0.01 to about 150 mg/kg/day, more typically from about 0.1 to about 15 mg/kg/day, and most typically from about 1 to about 10 mg/kg/day, , for example 5 mg/kg/day or 3 mg/kg/day

For other modes of administration, dosage amount and interval can be adjusted individually to provide plasma levels of the administered compound effective for the particular clinical indication being treated. For example, in one embodiment, a compound according to the invention can be administered in relatively high concentrations multiple times per day. Alternatively, it may be more desirable to administer a compound of the invention at minimal effective concentrations and to use a less frequent administration regimen. This will provide a therapeutic regimen that is commensurate with the severity of the individual's disease.

Utilizing the teachings provided herein, an effective therapeutic treatment regimen can be planned which does not cause substantial toxicity and yet is entirely effective to treat the clinical symptoms demonstrated by the particular patient. This planning should involve the careful choice of active compound by considering factors such as compound potency, relative bioavailability, patient body weight, presence and severity of adverse side effects, preferred mode of administration and the toxicity profile of the selected agent.

The compounds, compositions and methods of the present invention are further illustrated by the examples that follow. These examples are offered to illustrate, but not to limit the claimed invention.

EXAMPLES

Material and Methods

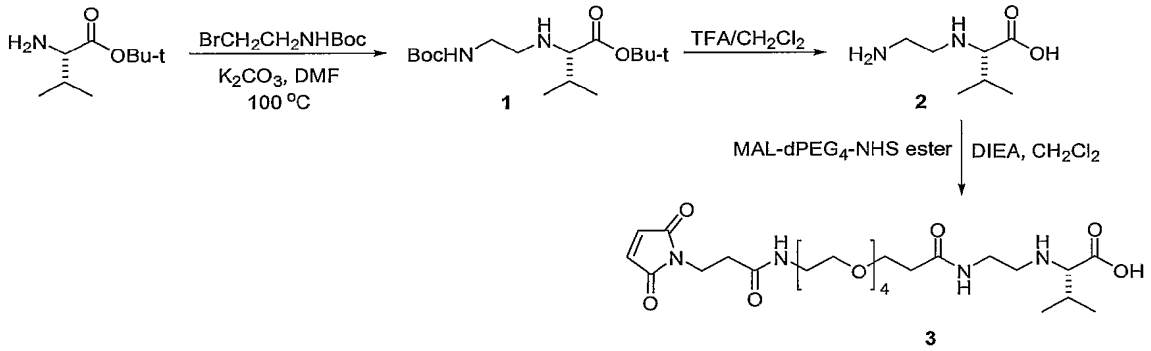
In the examples below, unless otherwise stated, temperatures are given in degrees Celsius (°C); operations were carried out at room or ambient temperature (typically a range of from about 18-25 °C; evaporation of solvent was carried out using a rotary evaporator under reduced pressure (typically, 4.5-30 mmHg) with a bath temperature of up to 60 °C; the course of reactions was typically followed by TLC and reaction times are provided for illustration only; melting points are uncorrected; products exhibited satisfactory ^{1}H-NMR and/or microanalytical data; yields are provided for illustration only; and the following conventional abbreviations are also used: mp (melting point), L (liter(s)), mL (milliliters), mmol (millimoles), g (grams), mg (milligrams), min (minutes), LC-MS (liquid chromatography-mass spectrometry) and h (hours).

^{1}H-NMR spectra were measured on a Varian Mercury 300 MHz spectrometer and were consistent with the assigned structures. Chemical shifts were reported in parts per million (ppm) downfield from tetramethylsilane. Electrospray mass spectra were recorded on a Perkin Elmer Sciex API 365 mass spectrometer. Elemental analyses were performed by Robertson Microlit Laboratories, Madison, NJ. Silica gel for flash chromatography was E. Merck grade (230-400 mesh). Reverse-Phase analytical HPLC was performed on either a HP 1100 or a Varian ProStar 210 instrument with a Phenomenex Luna 5 μm C-18(2) 150 mm x 4.6 mm column or a Varian Microsorb-MV 0.1 μm C-18 150 mm x 4.6 mm column. A flow rate of 1 mL/min was with either a gradient of 0% to 50% buffer B over 15 minutes or 10% to 100% buffer B over 10 minutes with detection by UV at 254nm. Buffer A, 20 mM ammonium formate + 20% acetonitrile or 0.1% trifluoroacetic acid in acetonitrile; buffer B, 20 mM ammonium formate + 80% acetonitrile or 0.1% aqueous trifluoroacetic acid. Reverse phase preparative HPLC were performed on a Varian ProStar 215 instrument with a Waters Delta Pak 15 μm C-18 300 mm x 7.8 mm column.

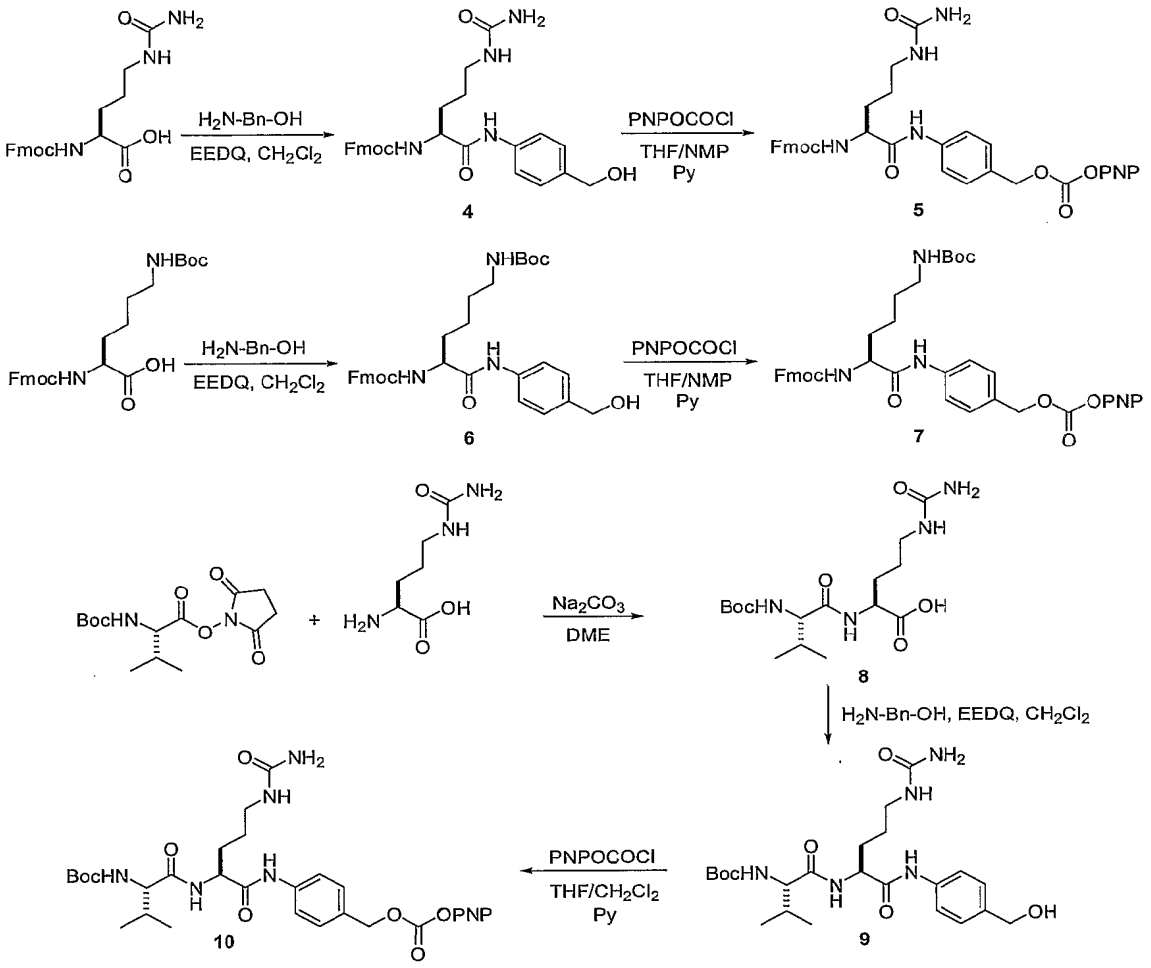
EXAMPLE 1: Synthesis of Peptide Linker Conjugates

1.1a Synthesis Methodology

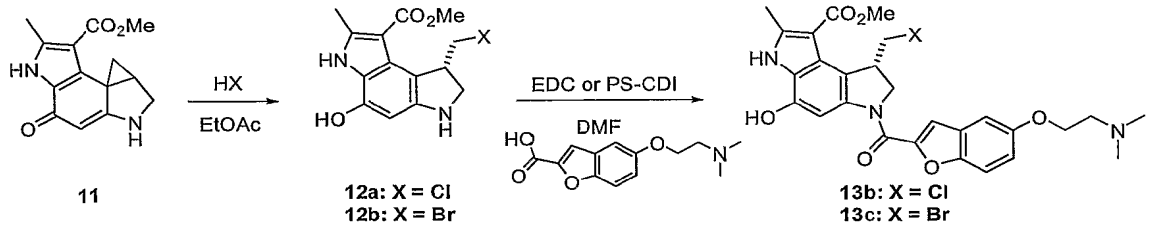
Scheme 1



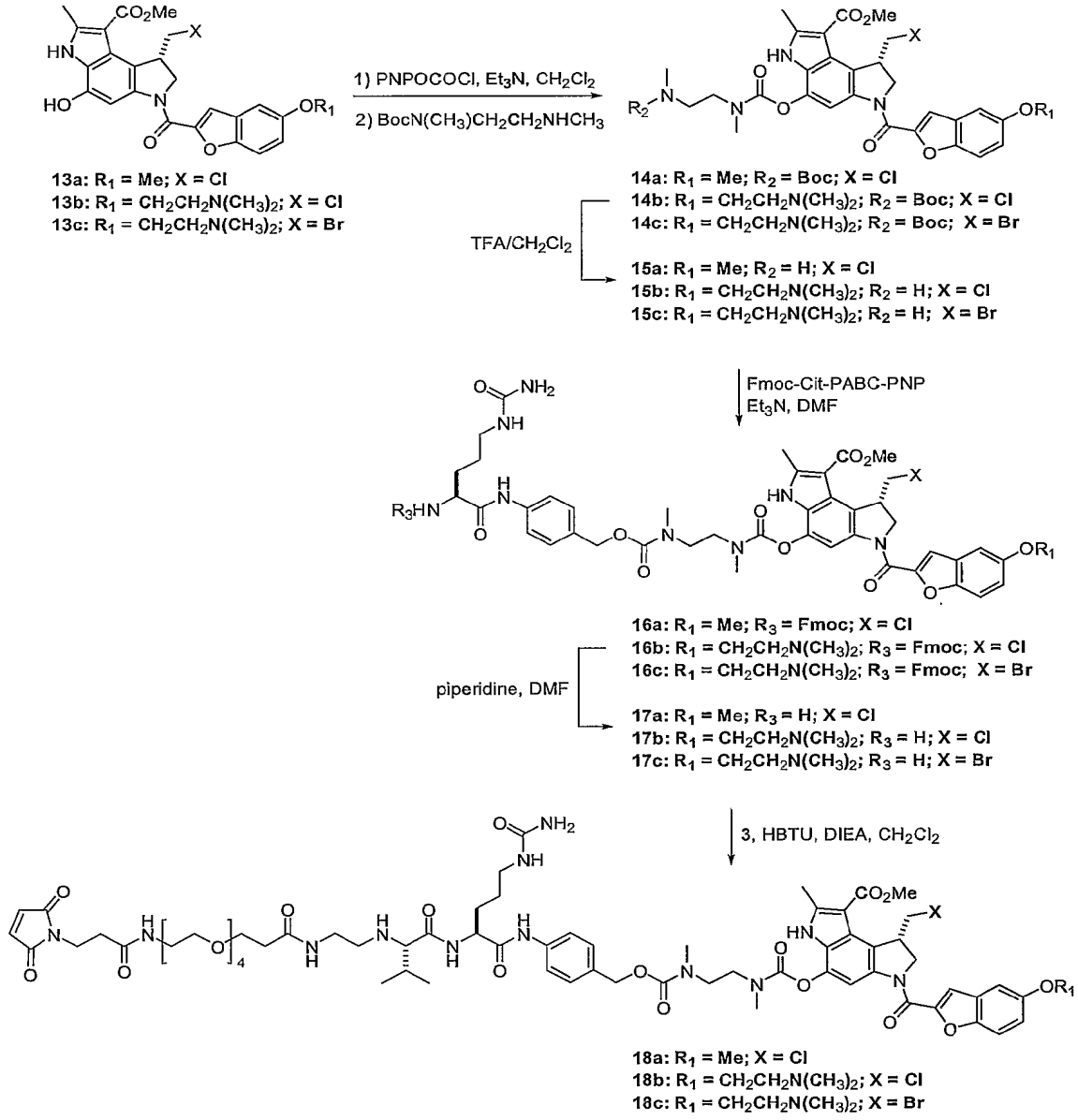
Scheme 2



Scheme 3



Scheme 4



13a: R_1 = Me; X = Cl
13b: R_1 = $CH_2CH_2N(CH_3)_2$; X = Cl
13c: R_1 = $CH_2CH_2N(CH_3)_2$; X = Br

14a: R_1 = Me; R_2 = Boc; X = Cl
14b: R_1 = $CH_2CH_2N(CH_3)_2$; R_2 = Boc; X = Cl
14c: R_1 = $CH_2CH_2N(CH_3)_2$; R_2 = Boc; X = Br

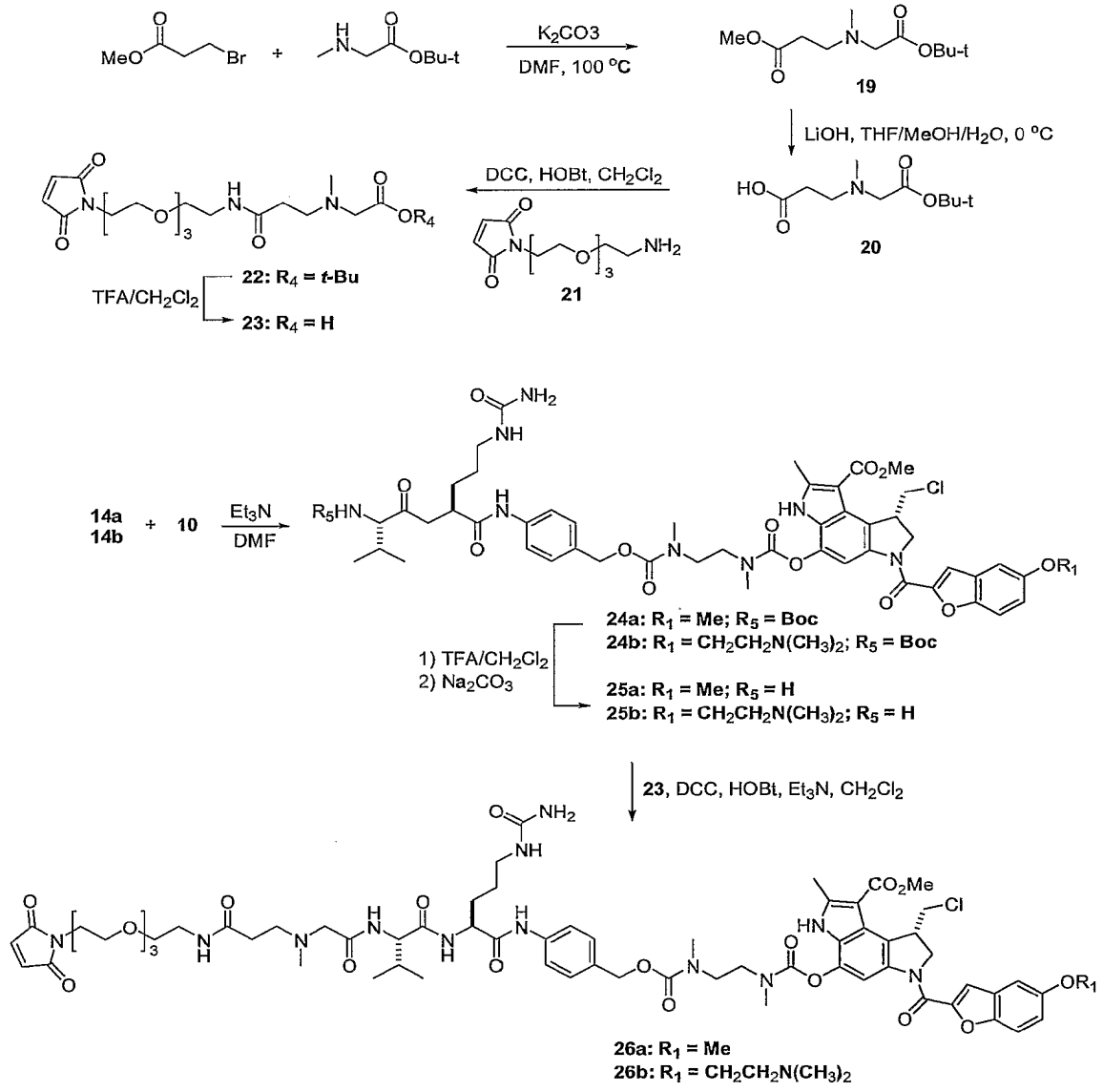
15a: R_1 = Me; R_2 = H; X = Cl
15b: R_1 = $CH_2CH_2N(CH_3)_2$; R_2 = H; X = Cl
15c: R_1 = $CH_2CH_2N(CH_3)_2$; R_2 = H; X = Br

16a: R_1 = Me; R_3 = Fmoc; X = Cl
16b: R_1 = $CH_2CH_2N(CH_3)_2$; R_3 = Fmoc; X = Cl
16c: R_1 = $CH_2CH_2N(CH_3)_2$; R_3 = Fmoc; X = Br

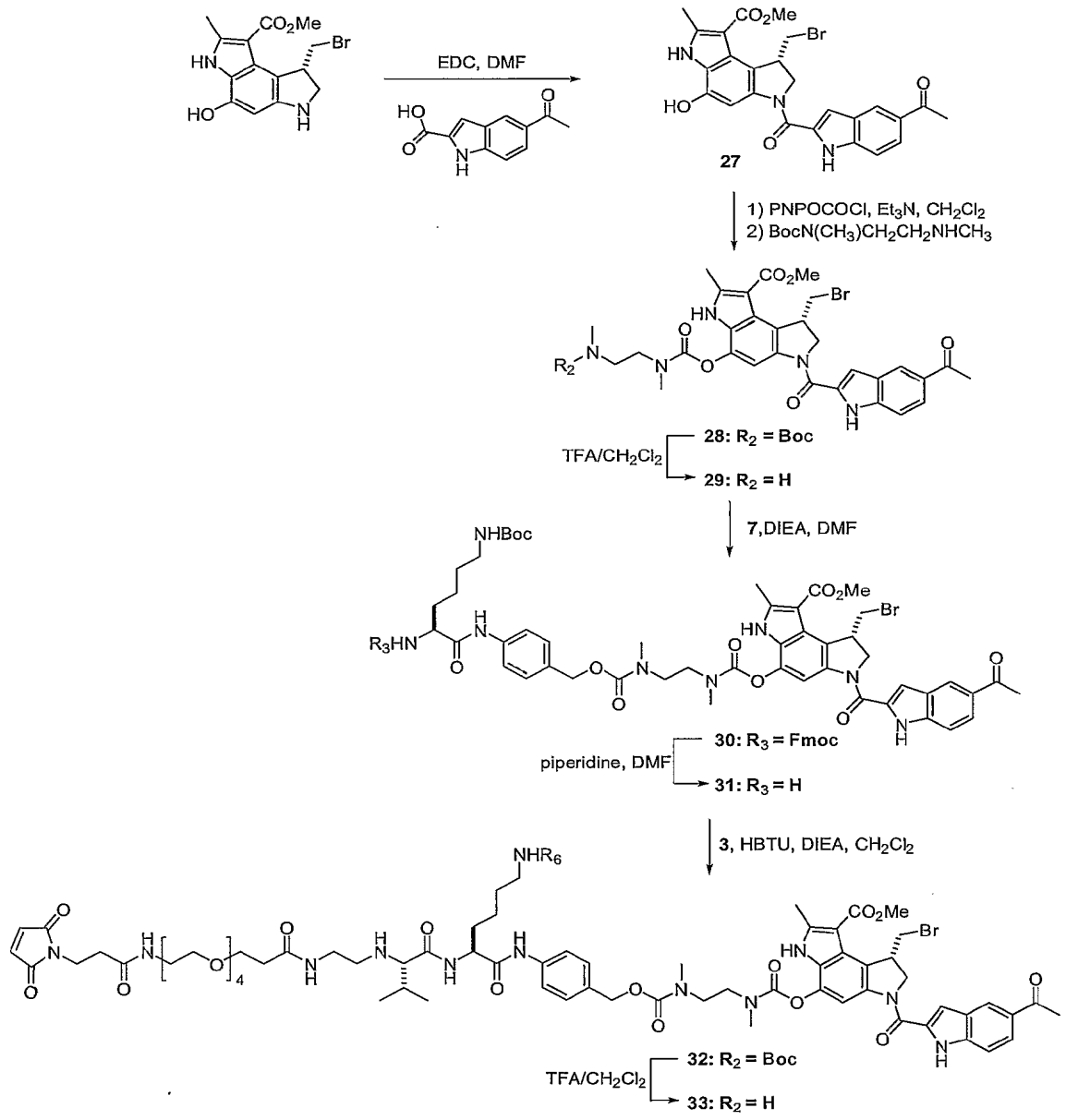
17a: R_1 = Me; R_3 = H; X = Cl
17b: R_1 = $CH_2CH_2N(CH_3)_2$; R_3 = H; X = Cl
17c: R_1 = $CH_2CH_2N(CH_3)_2$; R_3 = H; X = Br

18a: R_1 = Me; X = Cl
18b: R_1 = $CH_2CH_2N(CH_3)_2$; X = Cl
18c: R_1 = $CH_2CH_2N(CH_3)_2$; X = Br

Scheme 5


K2CO3
DMF, 100 °C
19
LiOH, THF/MeOH/H2O, 0 °C
20
DCC, HOBt, CH2Cl2
21
22: R4 = t-Bu
TFA/CH2Cl2
23: R4 = H
14a
14b
10
Et3N
DMF
24a: R1 = Me; R5 = Boc
24b: R1 = CH2CH2N(CH3)2; R5 = Boc
1) TFA/CH2Cl2
2) Na2CO3
25a: R1 = Me; R5 = H
25b: R1 = CH2CH2N(CH3)2; R5 = H
23, DCC, HOBt, Et3N, CH2Cl2
26a: R1 = Me
26b: R1 = CH2CH2N(CH3)2

Scheme 6



1.1b Synthesis of Compound 1: *N*-[2'-(*N*'-tert-butoxycarbonyl-animo)-ethyl]-valine tert-butyl ester. To a solution of 2-(*N*-tert-butoxycarbonyl-amino)-ethyl bromide (1 g, 4.5 mmole) and valine tert-butyl ester (0.936 g, 4.5 mmole) in DMF (10 mL) was added potassium carbonate (1.85 g, 13.5 mmole). The mixture thus obtained was stirred at 100°C overnight. The reaction

mixture was concentrated and the residue was purified by flash chromatography on silica gel with ethyl acetate/hexanes (3/7) as eluent to give the title compound as an oil (0.16 g, 12%). ^{1}H NMR ($CDCl_3$) δ 0.94 (ft, 6H), 1.44 (s, 9H), 1.473 and 1.475 (2s, 9H), 1.88 (m, 1H), 2.51 (m, 1H), 2.78 (m, 2H), 3.11 (m, 1H), 3.22 (m, 1H), 3.39 and 4.13 (2bt, 1H), 5.00 (bs, 1H) ppm; LC-MS (ESI) 205 ($M+H^+$-112), 261 ($M+H^+$-Bu), 317 ($M+H^+$).

1.1c Synthesis of Compound 2: *N*-(2-Aminoethyl)-valine. The compound **1** (137 mg, 0.43 mmole) was dissolved in a solution of TFA/dichloromethane (2 mL, 1/1) at room temperature. The mixture thus obtained was stirred at room temperature for 30 min. The reaction mixture was concentrated to dryness to give the title compound as an oil (0.18 g, 95%) ^{1}H NMR (CD_3OD) δ 1.07 and 1.16 (2d, 6H), 2.35 (m, 1H), 3.2 (m, 1H), 3.38 (m, 4H) ppm; LC-MS (ESI) 217 ($M+H^+$).

1.1d Synthesis of Compound 3. To a solution of maleamide-dPEG$_4$-NHS ester (61 mg, 0.16 mmole) in dichloromethane (2 mL) was added dropwise compound **2** (80.7 mg, 0.16 mmole) and diisopropylethylamine (55.5 μL, 0.32 mmole) in dichloromethane (1 mL). The mixture thus obtained was stirred overnight. The solvent were removed on the rotovap, and the residue was purified by flash chromatography on silica gel with dichloromethane, followed by 5% methanol in dichloromethane and finally 100% methanol as eluent to give the title compound as colorless oil (87 mg, 97%). ^{1}H NMR (CDCl3) δ 1.08 (dd, 6H), 2.25 (m, 1H), 2.49 (t, 2H), 2.52 (t, 2H), 3.10-3.79 (m, 25H), 6.82 (s, 2H) ppm; LC-MS (ESI) 559 ($M+H^+$)

1.1e Synthesis of Compound 4: Fmoc-Cit-PABOH. To a solution of Fmoc-Cit-OH (1.0 g, 2.52 mmole) and 4-aminobenzylalcohol (341 mg, 2.77 mmole) in dichloromethane (10 mL) and methanol (5 mL) was added 2- ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline[EEDQ] (1.24g, 5.04 mmole) in one portion. The mixture was stirred in the dark for 16 hours. The solvents were removed on the rotovap, and the white solid was triturated with ether (100 mL). The resulting suspension was sonicated for 5 min and then left to stand for 30 min. The white solid was collected by filtration, washed with ether and dried in vacuo (1.23g, 97%). ^{1}H-NMR (DMSO) δ 1.32 to 1.52 (m, 2H), 1.52 to 1.74 (dm, 2H), 2.86 to 3.06 (dm, 2H), 4.1 (M, 1H), 4.42 (d , 2H),

5.07 (t, 1H), 5.40 (bs, 2H), 5.97 (t, 1H), 7.19 to 7.95 (m, 12H), 8.10 (d, 1H), 9.97 (s, 1H) ppm; LC-MS (ESI) 503.1 ($M+H^+$).

1.1f Synthesis of Compound 5: Fmoc-Cit-PABC-PNP. To a solution of Compound **4** (309 mg, 0.62 mmole) and p-nitrophenylchloroformate (372 mg, 1.85 mmole) in Tetrahydrofuran (30 mL) and 1-methyl-2-pyrrolidine (1 mL) was added pyridine (100 µL, 1.23 mmole) in one portion. The mixture thus obtained was stirred at room temperature for 30 minutes. The solvents were removed on the rotovap, and the residue was purified by flash chromatography on silica gel with dichloromethane, followed by 3% methanol in dichloromethane and finally 10% methanol in dichloromethane as eluent to give the title compound as a white solid (97.9 mg, 70%). LC-MS (ESI) 668 ($M+H^+$).

1.1g Synthesis of Compound 6: Fmoc-Lys(Boc)-PABOH. Compound **6** was prepared as described above for Compound **4** in 98% yield. ^1H NMR (DMSO) δ 1.40 (s, 9H), 1.38 (m, 2H), 1.50 to 1.74 (dm, 2H), 3.04 (t, 2H), 3.30 (q, 3H), 4.19 to 4.31 (m, 2H), 4.41 (d , 2H), 4.55 (s, 2H), 7.28 to 7.68 (m, 12H), 8.00 (d, 1H) ppm; LC-MS (ESI) 574 ($M+H^+$).

1.1h Synthesis of Compound 7: Fmoc-Lys(Boc)-PABC-PNP. Compound **7** was prepared as described above for Compound **5** in 70% yield. ^1H NMR (CD_3Cl) δ 1.44 (s, 9H), 1.49-1.60 (m, 6H), 1.73 (m, 1H), 2.00 (m, 1H), 3.11 (m, 1H), 3.20 (bs, 1H), 4.23 (m, 2H), 4.46 (bs, 2H), 4.67 (bs, 1H), 5.56 (bs, 1H), 7.28 (m, 2H), 7.36-7.41 (m, 6H), 7.59 (m, 4H), 7.76 (d, 2H), 8.26 (dd, 2H), 8.45 (bs, 1H) ppm; LC-MS (ESI) 639 ($M+H^+$-Boc), 684 ($M+H^+$-Bu), 739 ($M+H^+$), 778 ($M+K^+$).

1.1i Synthesis of Compound 8: Boc-Val-Cit-OH. To a solution of Citrulline (2.54 g, 14.50 mmole) and Sodium Bicarbonate (1.28 g) in water (40 mL) was added Boc–Val–OSu (4.34 g, 13.81 mmole) dissolved in dimethoxyethane (DME). To aid the solubility of the mixture tetrahydrofuran (10 mL) was added. The mixture thus obtained was let stir overnight at room temperature. Aqueous citric acid (15%, 75 mL) was added and the mixture was extracted with 10% 2–propanol/ethyl acetate (2 x 100 mL). The organic layer was washed with brine (2 x 150

mL) and the solvents were removed on the rotovap. The resulting white solid was dried in vacuo for 5 hours and then treated with ether (100 mL). After brief sonication and trituration, the white solid product was collected by filtration (1.39 g, 27%). ^{1}H NMR (CD_3OD)δ 0.91 (dd, 3H), 0.98 (dd, 3H), 1.44 (s, 9H), 1.70 (m, 2H), 1.87 (m, 2H), 2.02 (m, 2H), 3.11 (t, 2H), 3.89 (t, 1H), 4.39 (q, 1H), 8.22 (d, 1H) ppm; LC-MS (ESI) 375 ($M+H^+$).

1.1j Synthesis of Compound 9: Boc-Val-Cit-PABOH. Compound **9** was prepared as described above for Compound **4** in 71% yield. ^{1}H NMR (CD_3OD) δ 0.93 and 0.97 (2d, 6H), 1.44 (s, 9H), 1.58 (m, 2H), 1.75 (m, 1H), 1.90 (m, 1H), 2.05 (m, 1H), 3.10 (m, 1H), 3.19 (m, 1H), 3.91 (d, 1H), 4.52 (m, 1H), 5.25 (s, 2H), 7.40 (d, 2H), 7.45 (dd, 2H), 7.64 (d, 4H), 8.29 (dd, 2H) ppm; LC-MS (ESI) 480 ($M+H^+$).

1.1k Synthesis of Compound 10: Boc-Val-Cit-PABC-PNP. A solution of Boc-Val-Cit-PABOH (178 mg, 0.370 mmole) in THF (8 mL) in CH_2Cl_2 (4 mL) was stirred at room temperature with PNP chloroformate (160 mg, 0.80 mmole) and pyridine (65 μL, 0.80 mmole) for 3 h. Ethyl acetate (100 mL) and 10% aqueous citric acid (50 mL) were added to the reaction mixture and organic layer was washed with brine, dried and concentrated and the residue was purified by flash chromatography on silica gel with 5% methanol in as eluent to give the title compound as a white solid (165 mg, 70%). ^{1}H NMR (CD_3OD) δ 0.93 (dd, 3H), 0.97 (dd, 3H), 1.44 (s, 9H), 1.58 (m, 2H), 1.75 (m, 1H), 1.89 (m, 1H), 2.05 (m, 1H), 3.10 (m, 1H), 3.20 (m, 1H), 3.90 (d, 1H), 4.51 (m, 1H), 4.55 (s, 2H), 7.29 (d, 2H), 7.55 (d, 2H) ppm; LC-MS (ESI) 545 ($M + H^+$ - Boc), 645 ($M + H^+$), 667 ($M + Na^+$), 683 ($M + K^+$).

1.1l Synthesis of Compound 12a. To a suspension of Compound **11** (20 mg, 0.078 mmole) in ethyl acetate (5 mL) was bubbled HCl gas for 20 min (by the time, the suspension became to a clean solution). The reaction mixture was stirred for additional 5 min then the mixture was concentrated to dryness to give the title compound as yellow solid (26 mg, 100%) which was used in next step without further purification. LC-MS (ESI) 260 ($M + H^+ - Cl$), 295 ($M + H^+$).

1.1m Synthesis of Compound 12b. To a suspension of Compound **11** (20 mg, 0.078 mmole) in ethyl acetate (5 mL) was bubbled HBr gas for 20 min (by the time, the suspension became to a

clean solution). The reaction mixture was stirred for additional 5 min then the mixture was concentrated to dryness to give the title compound as yellow solid (33 mg, 100%) which was used in next step without further purification. LC-MS (ESI) 260 ($M + H^+ - Br$), 339 ($M + H^+$), 341 ($M + H^+ + 2$).

1.1n Synthesis of Compound 13b. To a solution of Compound **12a** (26 mg, 0.078 mmole) in DMF (2 mL) were added 5-(2-dimethylamino-ethoxy)-benzofuran-2-carboxylic acid (44 mg, 0.155 mmole) and EDC (30 mg, 0.155 mmole). The mixture thus obtained was stirred at room temperature for 2 h. The mixture was concentrated and the residue was dissolved in $H_2O/CH_3CN/TFA$ (4/1.5/0.5, 6 mL) and it was placed in freezer for 3 h. A yellow solid was collected by filtration (35 mg, 85%). ^1H NMR (CD_3OD) δ 2.67 (s, 3H), 3.01 (s, 6H), 3.34 (m, 2H), 3.63 (ft, 1H), 3.89 (s, 3H), 3.91 (m, 1H), 4.41 (m, 3H), 4.54 (m, 1H), 4.65 (m, 1H), 7.20 (dd, 1H), 7.36 (d, 1H), 7.54 (s, 1H), 7.59 (d, 1H), 7.73 (bs, 1H), 11.75 (s, 1H) ppm; LC-MS (ESI) 490 ($M + H^+$ - Cl), 526 ($M + H^+$)

1.1o Synthesis of Compound 13c. To a solution of Compound **12b** (19 mg, 0.0387) in DMF (2 mL) were added 5-(2-dimethylamino-ethoxy)-benzofuran-2-carboxylic acid HBr salt (25 mg, 0.0775 mmole) and PS-carbodiimide (82 mg, mmole/g: 0.94, 0.0775 mmole). The reaction mixture was stirred at room temperature for 24 h. After filtration, the filtrate was concentrated and the residue was dissolved in $H_2O/CH_3CN/TFA$ (2/0.75/0.25, 3 mL) and it was placed in freezer for 3 h. The yellow solid was collected by filtration and dried to give the title compound (18 mg, 82%). LC-MS (ESI) 490 ($M + H^+$ - Br), 570 ($M + H^+$), 572 ($M + H^+ + 2$)

1.1p Synthesis of Compound 14a. To a suspension of Compound **13a** (48 mg, 0.10 mmole) in dichloromethane (4 mL) were added *p*-nitrophenyl chloroformate (80 mg, 0.40 mmole) and triethylamine (56 μL, 0.40 m mole) at –78 °C. The mixture was warmed up to room temperature slowly and the stirring was continued for additional 30 min. To the reaction mixture was added compound *N*-Boc-*N,N'*-dimethylethylenediamine (166 mg, 0.80 mmole) and stirred overnight. The mixture was concentrated and the residue was purified by flash chromatography on silica gel with 1.25% methanol in dichloromethane as eluent to give the title compound as a white solid

(71 mg, 100%) ^{1}H NMR δ 1.45-1.47 (m, 9H), 2.69 (s, 3H), 2.97 (s, 3H), 3.14-3.34 (m, 4H), 3.81-3.92 (m, 8H), 4.38-4.47 (m, 3H), 4.70 (d, 1H), 7.05 (dd, 1H), 7.11 (d, 1H), 7.45 (s, 1H), 7.48 (d, 1H), 7.99 (s, 1H), 10. 43 (s, 1H) ppm. LC-MS (ESI) 710 ($M-H^+$)

1.1q Synthesis of Compound 14b. To a suspension of Compound **13b** (48 mg, 0.075 mmole) in dichloromethane (2 mL) were added 4-nitrophenyl chloroformate (80 mg, 0.4 m mole) and triethylamine (40 mg, 0.4 m mole, 56 μL) at 0 °C. The mixture was warmed up to room temperature and stirring was continued additional 6 h. The solvent was evaporated and the residue was washed with ether to give the intermediate. The intermediate was dissolved in dichloromethane (2 mL) and to the reaction solution were added *N*-Boc-*N,N'*-dimethylethylenediamine (44 mg, 0.2 m mole) and triethylamine (20 mg, 0.2 mmole, 28 μL). The mixture thus obtained was stirred at room temperature overnight. The mixture was concentrated and the residue was purified by HPLC on C-18 column with ammonium formate (20 mM, pH 7.0) and acetonitrile as eluent to give the title compound as white solid (31 mg, 54%). LC-MS (ESI) 755 ($M+H^+$)

1.1r Synthesis of Compound 14c. To a suspension of Compound **13c** (24 mg, 0.04 mmole) in CH_2Cl_2 (2 mL) were added *p*-nitrophenyl chloroformate (64 mg, 0.32 mmole) and triethylamine (22 μL, 0.16 mmole) at 0 °C. The reaction mixture thus obtained was stirred at room temperature for 18 h. To the reaction mixture was added *N*-Boc-*N,N'*-dimethylethylenediamine (94 mg, 0.50 mmole) and the stirring was continued for additional 50 min. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with 5% methanol in dichloromethane as eluent to give the title compound as white solid (28 mg, 83%). LC-MS (ESI) 490, 570, 684 ($M + H^+$ - Boc), 784 ($M + H^+$), 805 ($M + Na^+$), 722 ($M + K^+$)

1.1s Synthesis of Compound 15a. Compound **14a** (70 mg, 0.10 m mole) was dissolved in trifluoroacetic acid (5 mL) and the mixture was stirred at room temperature for 30 min and concentrated to dryness and the product (72 mg, 100%) was used in next step without further purification. HPLC showed it to be >95% pure. ^{1}H NMR δ 2.64 (s, 3H), 2.93 (s, 3H), 3.19 (s, 3H), 3.30 (t, 1H), 3.79 (s, 3H), 3.85 (s, 3H), 3.81-3.85 (m, 1H), 4.27-4.49 (m, 3H), 4.59 (d, 1H),

4.68 (d, 1H), 6.97 (dd, 1H), 7.03 (d, 1H), 7.38 (s, 1H), 7.41 (d, 1H), 8.00 (br s, 1H), 10.61 (br s, 1H) ppm. LC-MS (ESI) 612 ($M+H^+$), 634 ($M+Na^+$)

1.1t Synthesis of Compound 15b. Compound **15b** was prepared as described above for Compound 15a in 100% yield. ^1H NMR (CD_3OD) δ 2.69 (s, 3H), 2.76 (s, 3H), 2.83 (bs, 1H), 3.01 (s, 6H), 3.08 (bs, 1H), 3.24 (bs, 2H), 3.42 (m, 2H), 3.63 (bs, 3H), 3.74 (bs, 1H), 3.91 (s, 3H), 3.92 (m, 1H), 4.40 (bs, 2H), 4.57 (bs, 2H), 4.71 (bs, 1H), 7.22 (bd, 1H), 7.36 (s, 1H), 7.56 (s, 1H), 7.59 (d, 1H), 8.04 (bs, 1H) ppm; LC-MS (ESI) 490, 526, 640 ($M+H^+$), 678 ($M+K^+$).

1.1u Synthesis of Compound 15c. Compound **15c** was prepared as described above for Compound **15a** in 100% yield. LC-MS (ESI) 490, 570, 684 ($M + H^+$), 722 ($M + K^+$)

1.1v Sythesis of Compound 16a. To a solution of Compound **5** (12.5 mg, 0.019 mmole) and Compound **15a** (10 mg, 0.014) in dimethylformamide (200 µL) was added triethylamine (6 µL, 0.044 mmole). The mixture thus obtained was stirred at room temperature overnight. Ether (5 mL) was added to the mixture and a white solid precipated out of solution. The solid was filtered and purified by flash chromatography on silica gel with dichloromethane, followed by 1% methanol in dichloromethane, 2% methanol in dichloromethane, 3% methanol in dichloromethane and finally 4% methanol in dichloromethane as eluent to give the title compound as a white solid (8.7 mg, 56%). LC-MS (ESI) 470, 1112 ($M+H^+$), 1134 ($M + Na^+$), 1150 ($M + K^+$)

1.1w Synthesis of Compound 16b. To a solution of Compound **15b** (5 mg, 0.0056 mmole) in DMF (0.35 mL) were added Compound **5** (3.8 mg, 0.0056 mmole) and DIEA (2 µL, 0.011 mmole). The mixture thus obtained was stirred at room temperature for 5 h. The mixture was concentrated and the residue was purified by flash chromatography on silica gel with 10% methanol in dichloromethane as eluent to give the title compound as a solid (3 mg, 45%). LC-MS (ESI) 490, 526, 1169 ($M + H^+$), 1208 ($M + K^+$)

1.1x Synthesis of Compound 16c. Compound **16c** was prepared as described above for Compound **16b** in 50% yield. LC-MS (ESI) 490, 570, 1212 ($M + H^+$), 1250 ($M + K^+$)

1.1y Synthesis of Compound 17a. To a solution of Compound **16a** (8.7 mg, 0.008 mmole) in dimethylformamide (500 µL) was added piperidine (100 µL) in one portion. The mixture thus obtained was stirred for 20 minutes at room temperature. The solvent were removed on the rotovap, and placed on the high vacuum for 1.5 h. The residue was take up in the minimal amount of dichloromethane (100 µL) and hexane (3 mL) was add to the solution, a white solid crashed out of solution which was filtered off and dried (6.7 mg, 96.7%). MS (ES) 470, 890.1 ($M+H^+$), 912 ($M + Na^+$), 928 ($M + K^+$).

1.1z Synthesis of Compound 17b. Compound **17b** was prepared as described above for Compound **17a** in 95% yield. LC-MS (ESI) 947 ($M + H^+$)

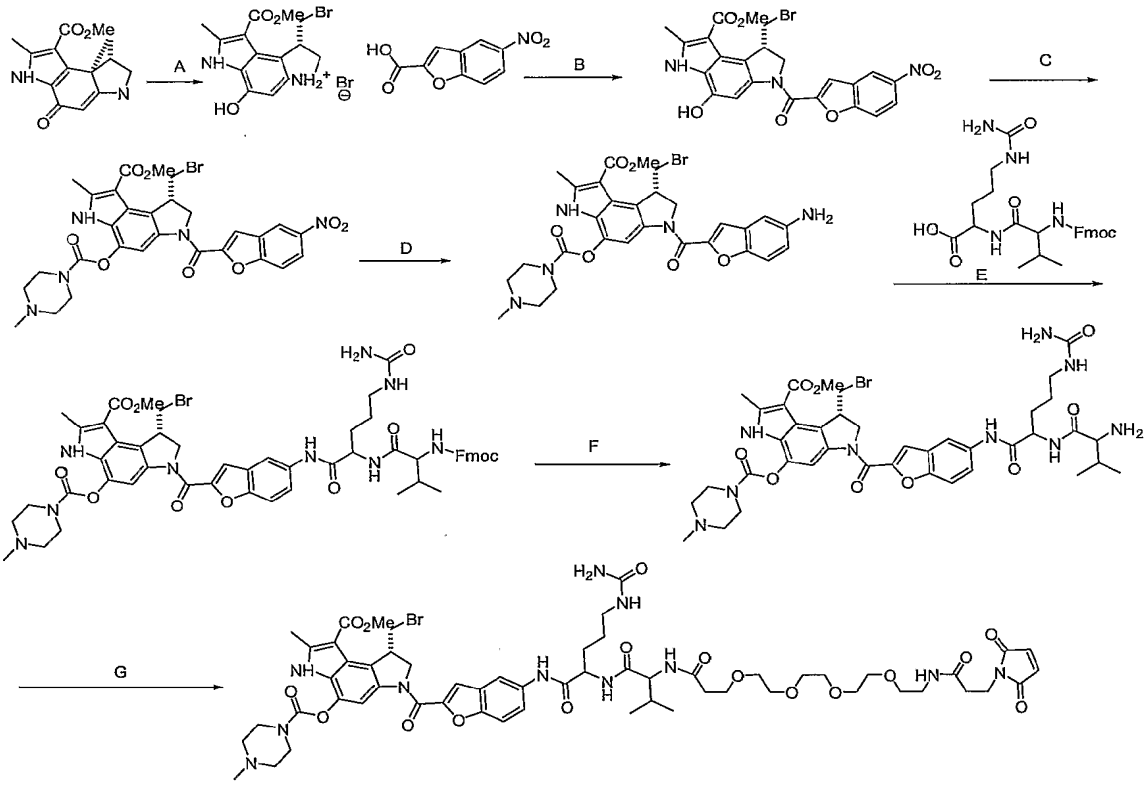
1.1aa Synthesis of Compound 17c. Compound **17c** was prepared as described above for Compound **17a** in 95% yield. LC-MS (ESI) 1015 ($M + H^+$)

1.1bb Synthesis of Compound 18a. To a solution of Compound **17a** (4.2 mg, 0.005 mmole) and Compound **3** (2.64 mg, 0.005 mmole) in dichloromethane (1 mL) was added in one portion PyBOP (3.7 mg, 0.007 mmole) followed by diisopropylethylamine (1 µL). The mixture thus obtained was stirred overnight at room temperature. The solvents were removed on the rotovap. The residue was purified by Prep HPLC to yield a beige solid (2.6 mg, 38.7%). MS (ES) 470, 1431 ($M + H^+$), 1453 ($M + Na^+$), 1469 ($M + K^+$)

1.1cc Synthesis of Compound 18b. To a solution of Compound **17b** (2.2 mg, 0.0025 mmole) and Compound **3** in 5% methanol in dichloromethane (400 µL) were added HBTU (9 mg, 0.0046 mmole) and DIEA (1.4 µL, 0.0046 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified on semi-preparative HPLC with 10 mM ammonium formate and acetonitrile as eluent to give the title compound as an oil (1.1 mg, 30%). LC-MS (ESI) 490, 526, 1488 ($M + H^+$), 1527 ($M + K^+$)

1.1dd Synthesis of Compound 18c. To a solution of Compound **17c** (6.5 mg, 0.0065 mmole) and the Compound **3** (5.5 mg, 0.0097 mmole) in 5% methanol in dichloromethane (0.5 mL) were added HBTU (3.7 mg, 0.0097 mmole) and DIEA (3.4 μL , 0.0194 m mole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 30 % methanol in dichloromethane as eluent to give the title compound as an oil (4 mg, 30%). LC-MS (ESI) 1532 ($M+H^+$), 1554 ($M+Na^+$), 1570 ($M+K^+$).

1.2 Synthesis methodology for duocarmycin-containing peptide linker without self-immolative spacer



1.2a Reaction A: To a suspension of Alkylating core 7 mg in 2mL of Ethyl Acetate was passed a slow stream of dry HBr gas until a clear solution is formed which took approximately 15 minutes. The reaction mixture was concentrated and dried overnight under high vacuum.

1.2b Reaction B: To a suspension of the bromo methyl seco compound prepared in step A in DMF was added EDC (10mg, 0.054mMoles) and 5-Nitro benzofuran carboxylic acid (12mg, 0.054 mMoles) and allowed to stir for 6 hours. To this reaction mixture was then added ethyl acetate and brine. The combined organic layers were concentrated after three extractions with ethyl acetate. And filterd over silica gel using MeOH/DCM with increasing amounts of MeOH The product was confirmed by Mass Spec, M+1 = 530

1.2c Reaction C: The 4'-OH was protected using methyl pipirazine carbonyl chloride (11 mg, 0.054 mMoles) in 2mL DCM, 200 µL Allyl alcohol and pyridine (21 µL) for 2 hours. The product was purified by silica gel column chromatography and Identified by Mass Spec, MS+1 = 654

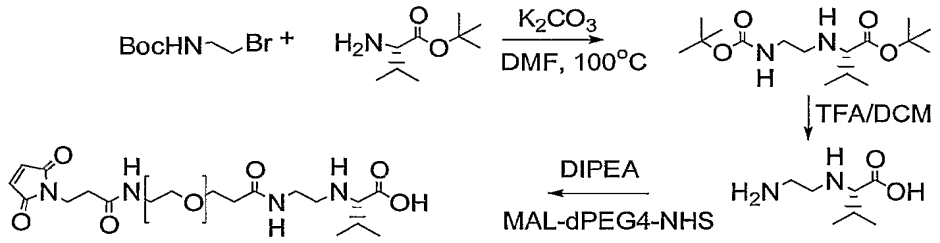
1.2d Reaction D: Reduction of Nitro group was done by hydogenolysis over Pd/C in DCM/MeOH (2:1) under 40 PSI for 45 minutes. The product was filtered and the filtrate concentrated and dried under high vacuum. The product was confirmed by mass spec analysis MS+1 = and carried out to the next step without further purification.

1.2e Reaction E: To a solution of above compound (18mg, 0.024 mMoles) in MeOH/DCM (2:1, 3 mL) was added Fmoc-Val-Citruline (29 mg, 0.06 mMoles) the resultant mixture was stirred for 10 minutes until all the acid dissolved. 15 mg, 0.06 moles of EEDQ was added and the reaction mixture was stirred in the dark overnight. The reaction mixture was then concentrated, rinsed with diethyl ether and the residue was purified by reverse phase Prep HPLC to give the product which was identified by Mass Spec M+1 = 1103.

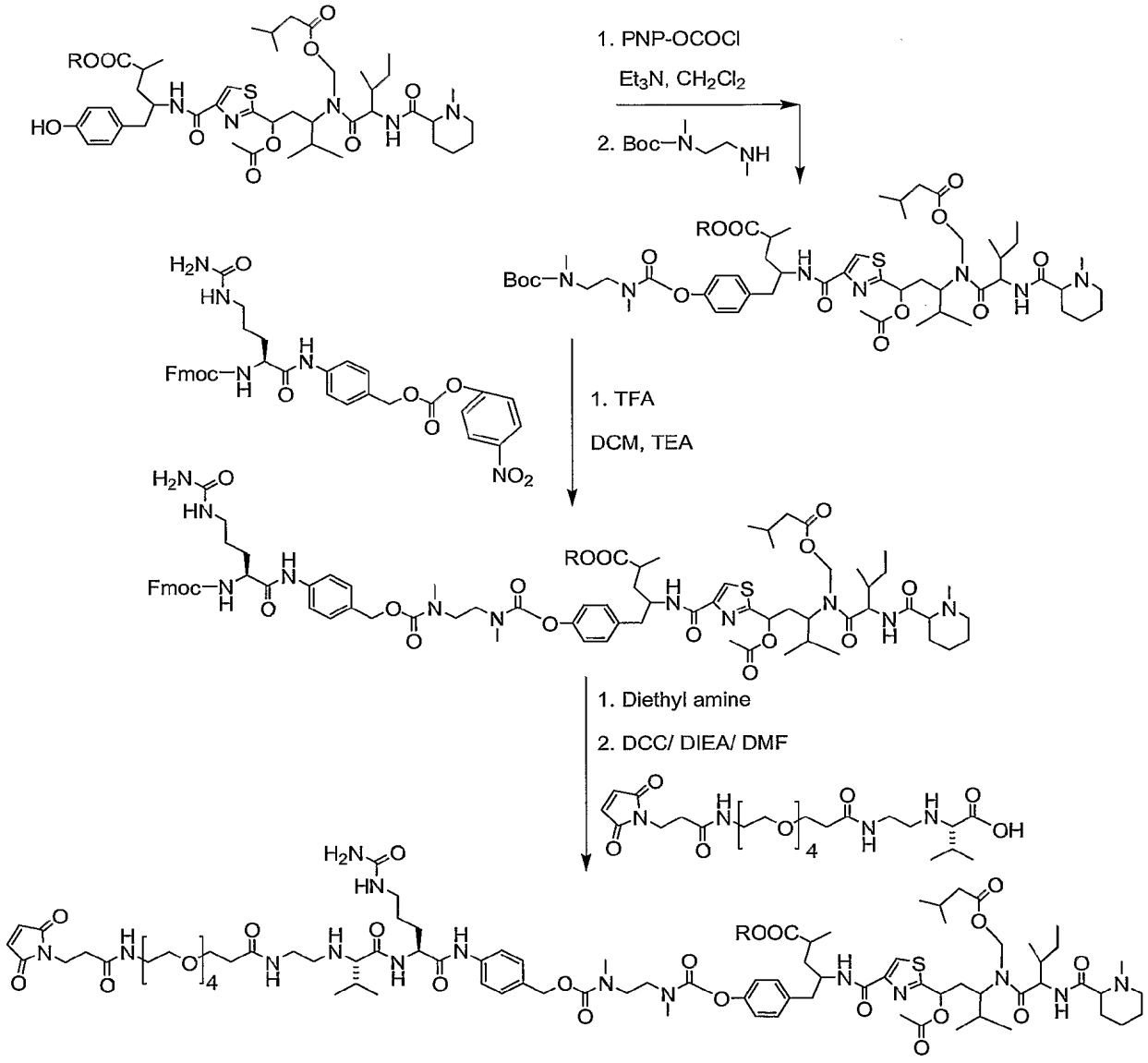
1.2f Reaction F: Deprotection of Fmoc protecting group was done using 5% pipiridine in 1 mL DMF for 10 minutes. Concentration of the reaction mixture was followed by rinsing the solid residue with diethyl ether. Product was confirmed by Mass Spec, MS+1 = 880 and M+K= 919

1.2g Reaction G: To a solution of the free amine in DMF (1.5 mL) prepared in step F was added Mal-$(PEG)_4$-NHS-ester (20 mg) and the reaction mixture stirred for 1 hr. Concentration followed by purification reverse phase Prep HPLC gave 2.8 mg of (11 % overall yield, beginning from Alkylating core) which was confirmed by mass spec MS+1 = 2178, M+Na = 1300 and M+K = 1316

1.3 Synthesis of Peptide Linker conjugated with Tubulysine A



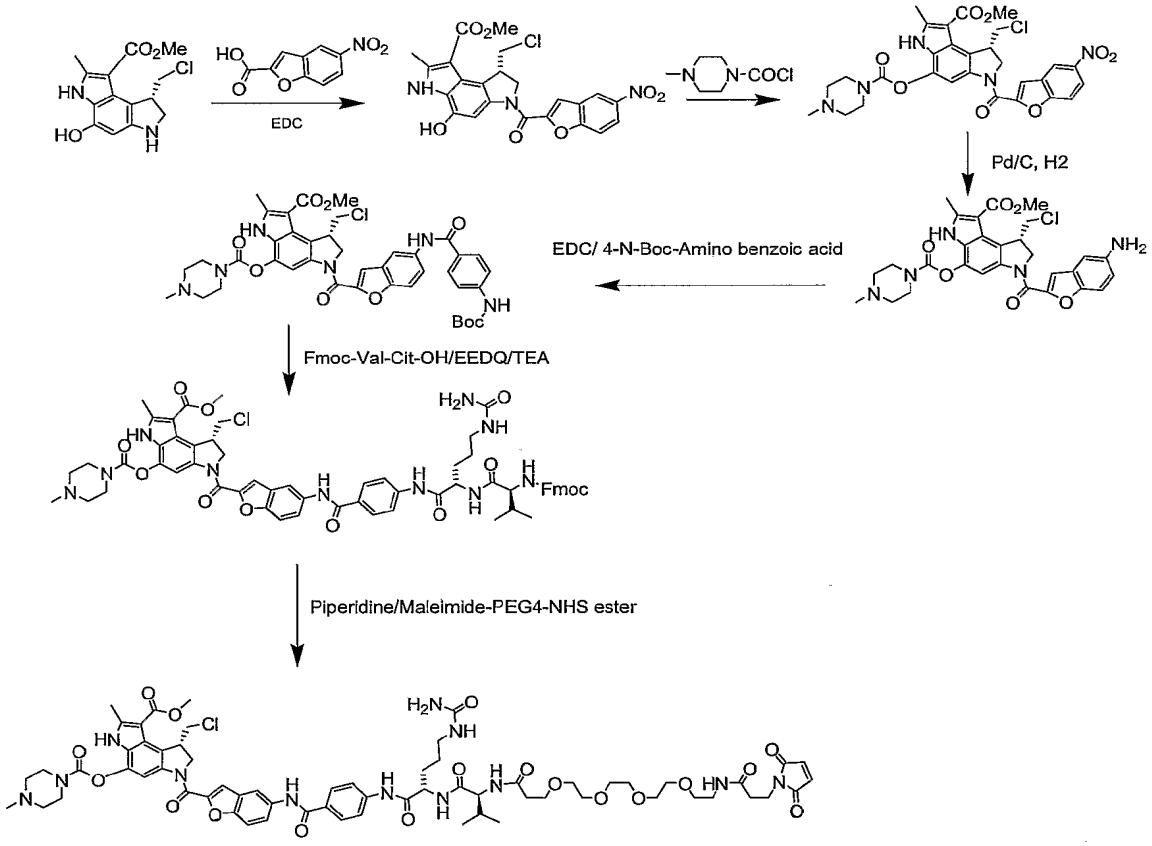
The ligand can be linked to PEG and peptide linker by the synthesis shown.



The synthesis of intermediates and ligand-drug conjugate having a peptide linker where the drug is Tubulysine A is shown hereinabove. This basic method may be used with other drugs.

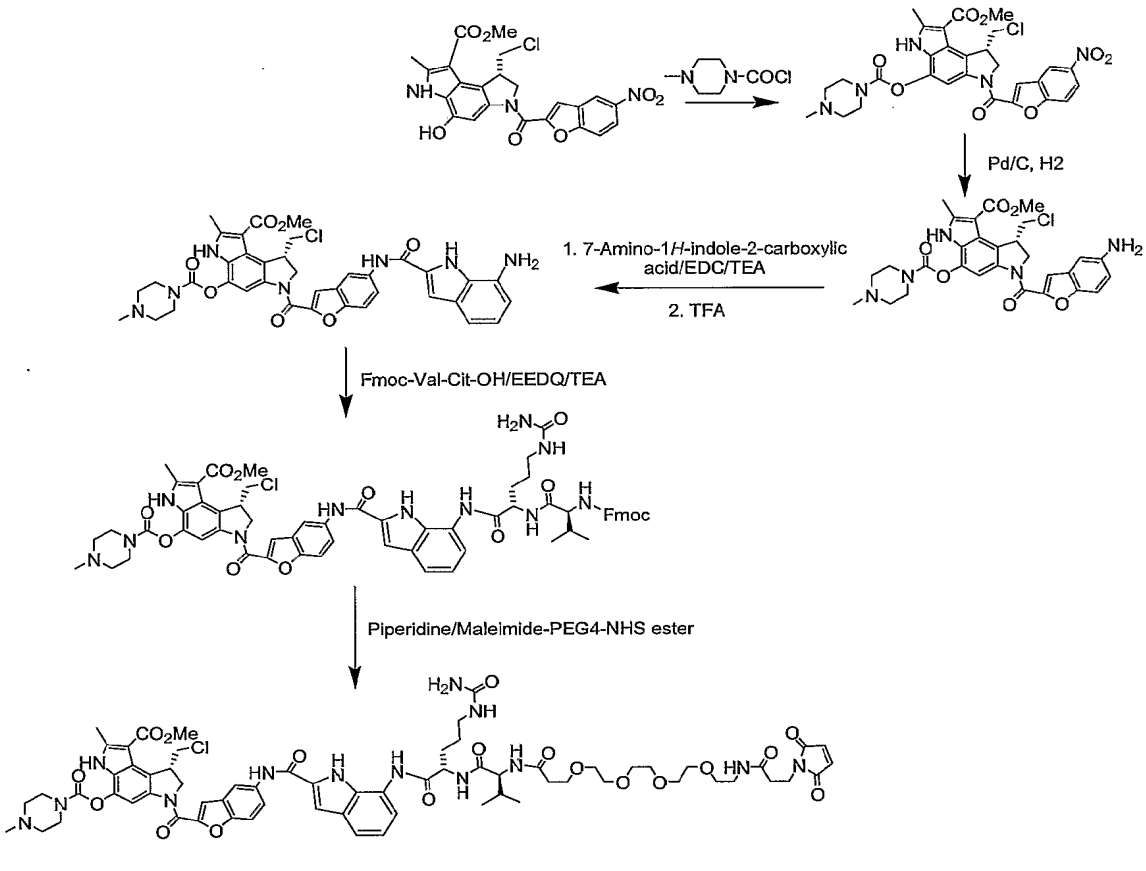
5

1.4a Synthesis of peptide-linker conjugate 111

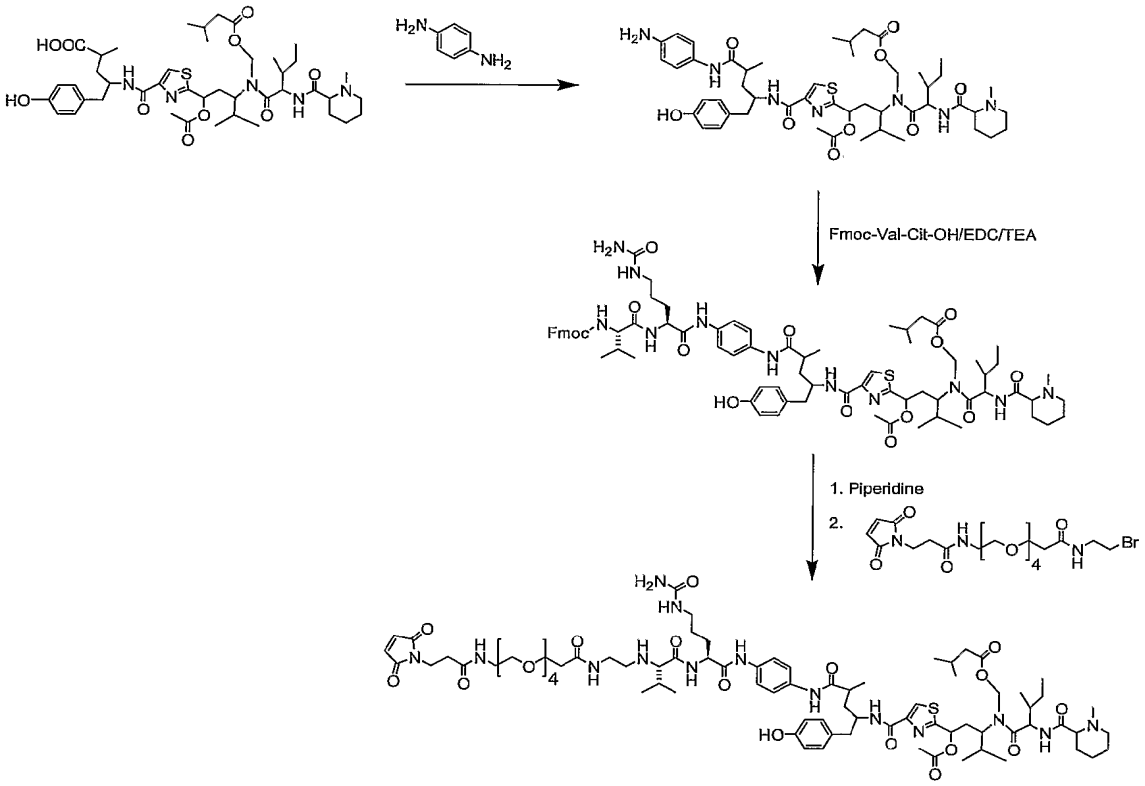


111

1.4b Synthesis of peptide-linker conjugate 112



5 1.4c Synthesis of peptide-linker conjugate 113

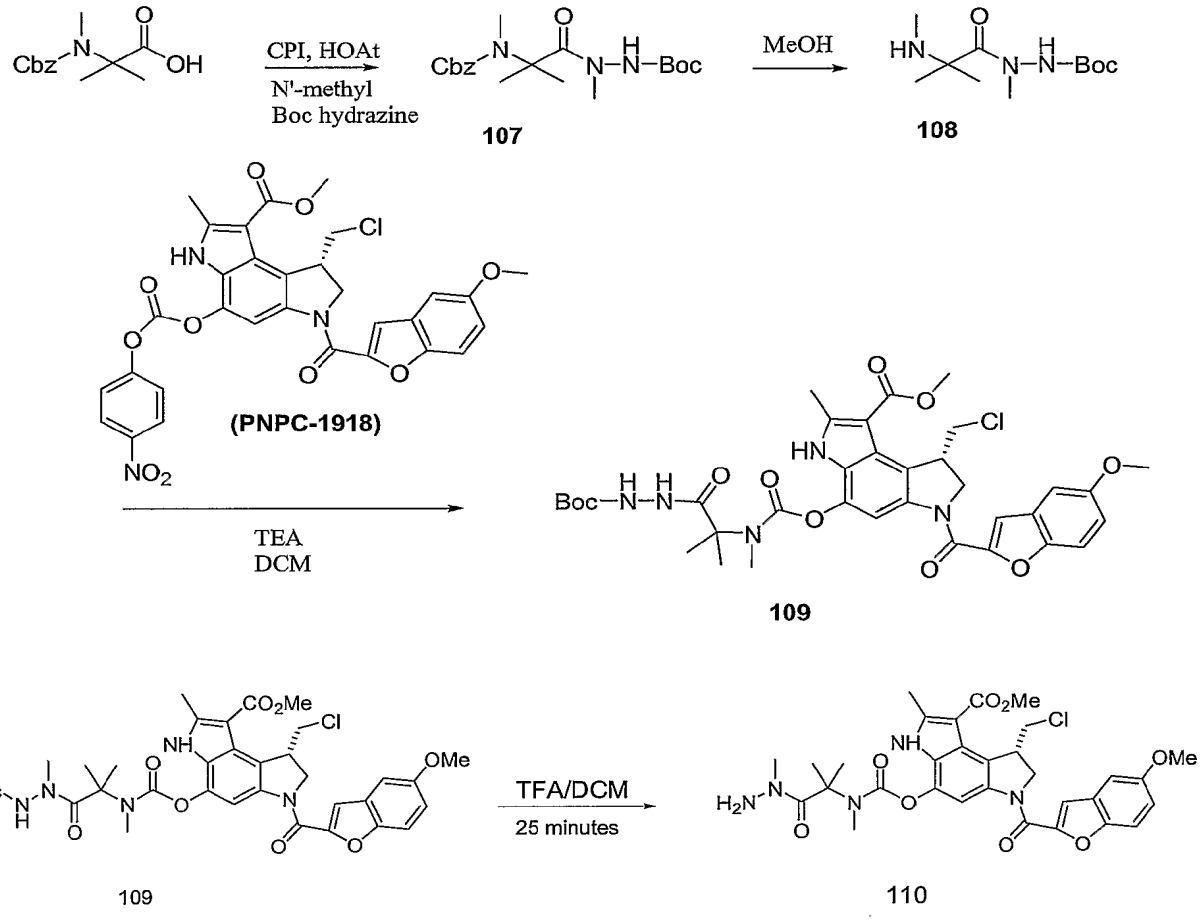


113

EXAMPLE 2: Synthesis of 6-Membered Hydrazine Linker Conjugates

2.1 Synthesis of a 6-Membered *gem*-dimethyl hydrazine linker conjugated to a duocarmycin derivative cytotoxin

2.1a Synthesis Scheme for Compound 109



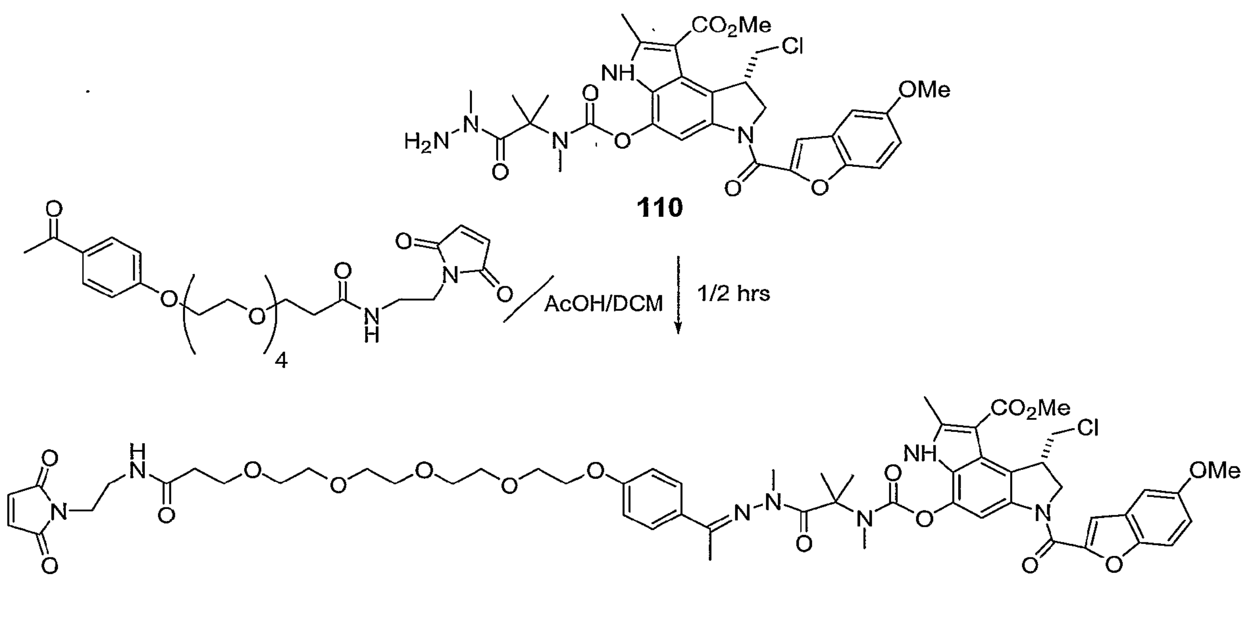
2.1b Synthesis of Compound 110

To a suspension of Cbz-dimethyl alanine (1g, 3.98 mMoles) in 30 mL of DCM at ice-bath temperature was added HOAT (catalytic, 0.25 equivalents), DIPEA (2.8 mL, 16 mmoles) followed by 2-chloro-1,3-dimethylimidazolidinium hexafluorophosphate (CIP) (1.2 g, 4.4

mmoles). To this reaction mixture was then added Boc-NN(Me) (643 moles, 4.4 mmoles). The reaction mixture was allowed to stir overnight at room temperature. To the reaction mixture is added 10 % citric acid solution (100 mL) and extracted with DCM. The organic phase was washed with water and then with a saturated solution of sodiumbicarbonate followed by water again. The organic phase was then concentrated and purified by silica gel column with increasing polarity of ethyl acetate in hexanes to give 860 mg , 57 % yield 107 which identified by mass spec M+1 = 380 and M+NH_4^+= 397.

The Cbz protecting group was removed by catalytic hydrogenation using Pd/C in MeOH to give compound 108 which was confirmed by MS.

To a solution of PNPC-1918 (10 mg, 0.1 mmoles) in 2 mL DCM was added drop wise a solution of Compound 108 (60 mg, 0.25 mmoles) in 8mL of DCM and the reaction mixture was allowed to stir for 2 days till all the starting material had disappeared. The reaction mixture was filtered through a short silica gel pad and then concentrated and purified by reverse phase Prep HPLC to give 4.2 mg of Compound 109. This was identified by Mass Spec M+1 = 740. Boc Deprotection of Compound 109 was done with pure TFA for 20 minutes to give Compound 110. The product was identified by Mass Spec, M+1 = 640.

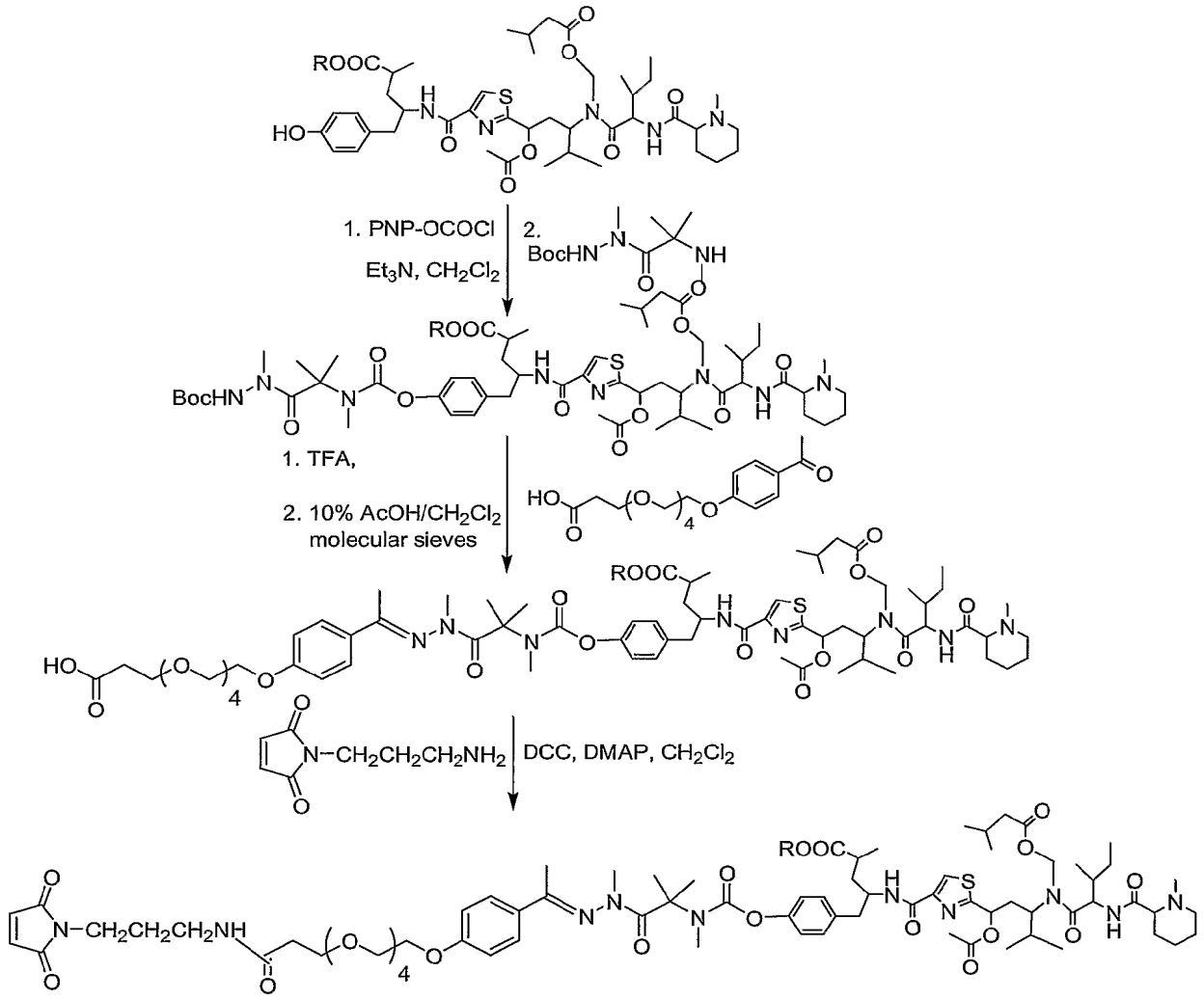


111

2.1c Synthesis of Compound 111

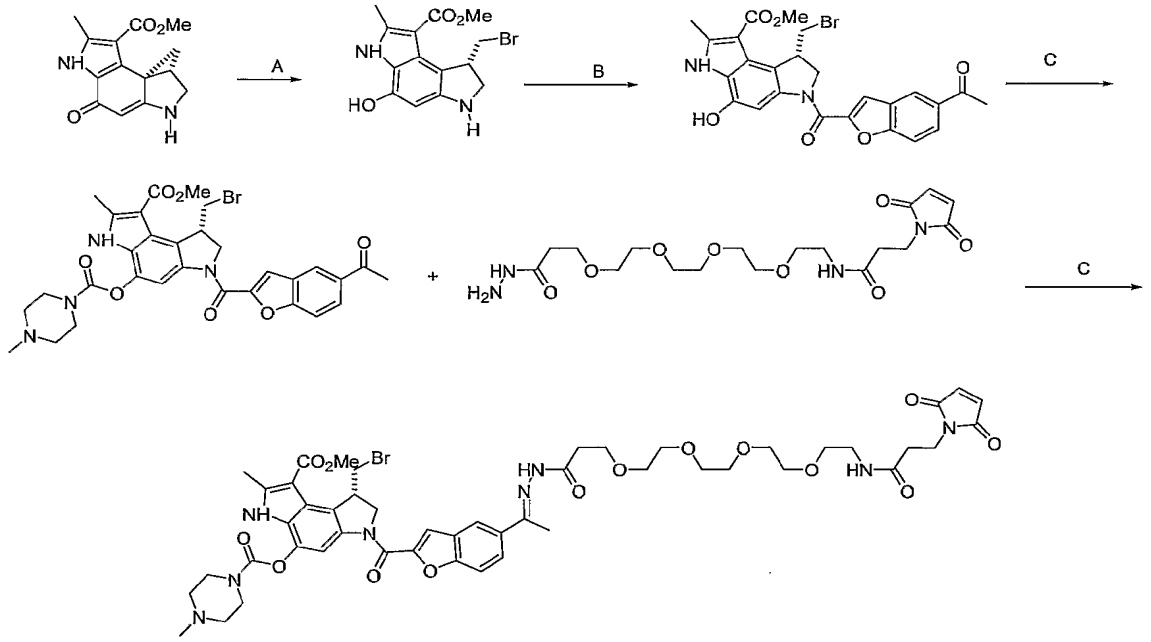
The Mal-PEG_4-Acetophenone and compound 110 (3mg, .005 mmoles) were combined concentrated and dried overnight under high vacuum. To this mixture was added a 1 mL of 5 % acetic acid solution prepared a day earlier and dried over molecular sieves. The formation of hydrazone was complete in less then an hour. After which the reaction mixture was concentration and purified by reverse phase Prep HPLC (ammonium formate *P*h = 7) to give 2.8 mg of compound 111 (60 % yield). The product was identified by Mass Spec, MS+1 = 1129, M+ NH_4 = 1146 and M+K = 1168

2.2 Synthesis of a *gem*-dimethyl 6-membered hydrazine linker conjugated to a tubulysin cytotoxin



Similar methodology as shown in Example 2.1 can be applied for the synthesis of a geminal dimethyl 6-membered hydrazine linker complexed with a drug such as tubulysin A is shown.

2.3 Synthesis of a hydrazine linker conjugated to a duocarmycin analog



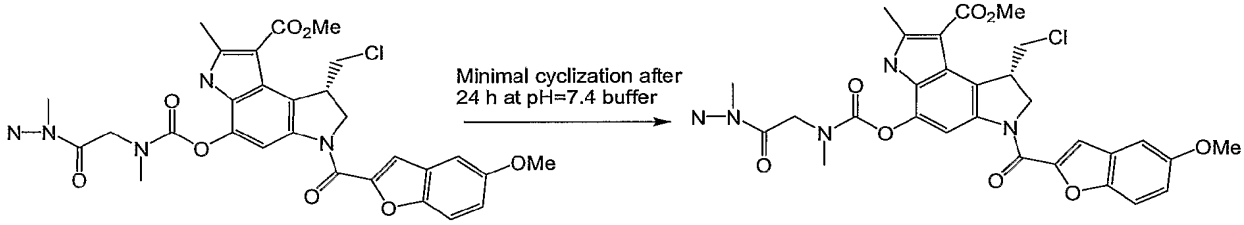
To a solution of the bromo methyl seco compound (0.074 mMoles) in 3 mL DMF was added the 5-actyl indole-2-carboxylate (30 mg, 0.15 mMoles) and EDC (28 mg, 0.15 mMoles) and the resulting mixture was stirred overnight. The reaction mixture was concentrated and purified by silica gel chromatography using 5% MeOH in DCM Tt give 29 mg (74 % yield) of product which was confirmed by mass spec M+1 = 523.

To a solution of the compound synthesized in step C in 5 mL DCM and 300 µL allyl alcohol was added methyl piperazine carbonyl chloride (22 mg, 0.11 m Moles) and pyridine 44 µL. The reaction mixture was stirred at room temperature for 5 hours. Concentration followed by purification by silica gel chromatography using 5 % MeOH/DCM as eluant gave 48 mg of the desired product (73 % yield). The product was confirmed by Mass Spec. M+1 = 650.

A solution of the above compound (8.2 mg , 0.012 mmoles) and Mal-PEG_4-hydrazine in 5 % acetic acid in anhydrous DCM was stirred at room temperature for 20 minutes followed by evaporation of Solvents and Reverse phase Prep HPLC using acetonitrile and ammonium formate buffered aqueous phase gave 2.5 mg of the desired final product which was confirmed by mass Spec, M+1 = 1063

2.4a Rate of cyclization of a dimethyl 6-membered hydrazine linker

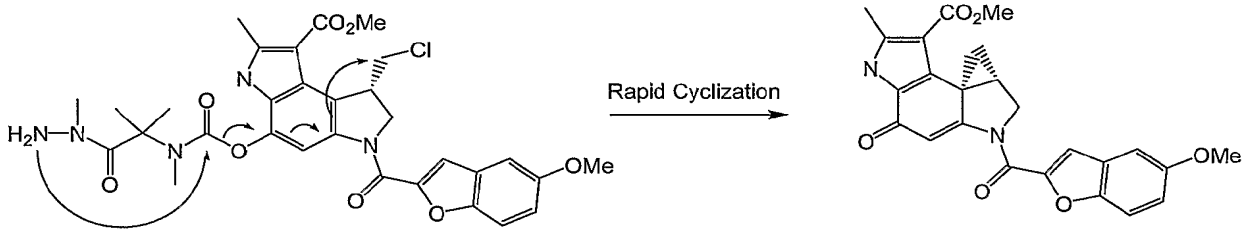
A duocarmycin analog conjugated to a dimethyl 6-membered hydrazine linker was incubated in buffer at pH 7.4 for 24 hours and the generation of cyclized product resulting from cyclization of the hydrazine linker, thereby releasing free duocarmycin analog, was assessed over time.



Minimal amounts of cyclized product were detected over 24 hours at pH=7.4, indicating this form of 6-membered hydrazine linker exhibits a relatively slow rate of cyclization.

2.4b Rate of cyclization of a *gem*-dimethyl 6-membered hydrazine linker

A duocarmycin analog conjugated to a *gem*-dimethyl 6-membered hydrazine linker was incubated in buffer at pH 7.4 and the generation of cyclized product resulting from cyclization of the hydrazine linker, thereby releasing free duocarmycin analog, was assessed over time.

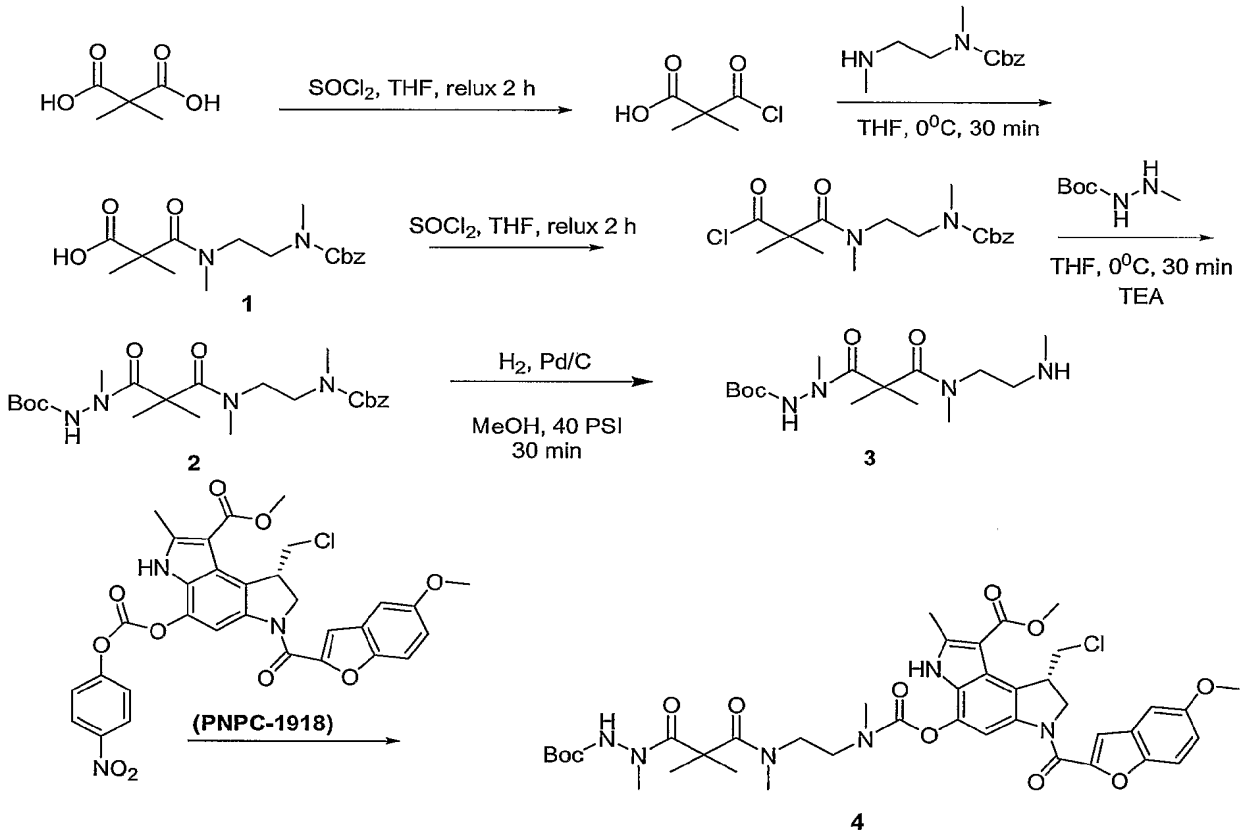


With the 6-membered *gem*-dimethyl linker, the cyclization reaction was quite rapid, proceeding to completion within a few minutes. Thus, the rate of cyclization for the *gem*-

dimethyl 6-membered hydrazine linker proceeded at a much faster rate than that of the 6-membered linker that did not contain the *gem*-dimethyl moiety.

EXAMPLE 3: Synthesis of 5-Membered Hydrazine Linker Conjugates

3.1 Synthesis Methodology for Compound 4



Cbz-DMDA-2,2-Dimethylmalonic acid (**1**)

To a solution of of 2,2-Dimethyl-malonic acid (2.0 gm, 0.0151 moles), Thionyl chloride (1.35 ml, 0.0182 moles) in THF (15 ml) in a 25 mL flask equipped with a stir bar, temperature probe, and reflux condenser was added a drop of DMF and the reaction mixture was heated to reflux for 2 hrs then cooled to room temperature. This reaction mixture was transferred to drop

wise to a solution of Cbz-DMDA (4 gm, 0.0182 moles) and triethylamine (4 ml, 0.0287 moles) in THF (5 ml) at 0 C and was stirred for 30 min at this temperature. The solvent was removed in vacuo and the residue dissolved in 1N HCl (50 ml) and extracted with DCM (2 x 25 ml). The combined organic layers were extracted with 1N NaOH (2 x 25 ml) and the combined aqueous layer were acidified (pH<1) with conc. HCl and extracted with EtOAc (2 x 25 ml), dried over $MgSO_4$, filtered and concentrated in vacuo to an off-white sticky solid, 3.44 gm, 68% yield. Compound **1** was confirmed by mass spec: *m/z* 337.0 $[M + 1]^+$
HPLC retention time: 3.77 min (mass spec)

Cbz-DMDA-2,2-Dimethylmalonic- Boc -*N*'-methylhydrazine (**2**)

To a solution of Compound **1** (3.0 gm, 0.0089 moles), Thionyl chloride (0.78 ml, 0.0107 moles) in THF (25 ml) in a 50ml 3N RBF equipped with a stir bar, temperature probe, and reflux condenser was added a drop of DMF and the reaction mixture was refluxed for 2 hrs then cooled to room temperature. This reaction mixture was then added dropwise to a solution of Boc-N-methyl hydrazine (1.33 gm, 0.091 moles) and triethylamine (3 ml, 0.0215 moles) in THF (25 ml) at 0 C and stirred for 30 min. The solvent was removed in vacuo and the residue dissolved in EtOAc(50 ml), dried over $MgSO_4$, filtered and concentrated in vacuo to a brown oil. The oil was dissolved in EtOAc and purified by column chromatography (100 % EtOAc) resulting in 3.45 gm, 83% yield of a clear oil. Compound **2** was confirmed by mass spec: *m/z* 465.2 [M + 1]
HPLC retention time: 3.97 min (mass spec)

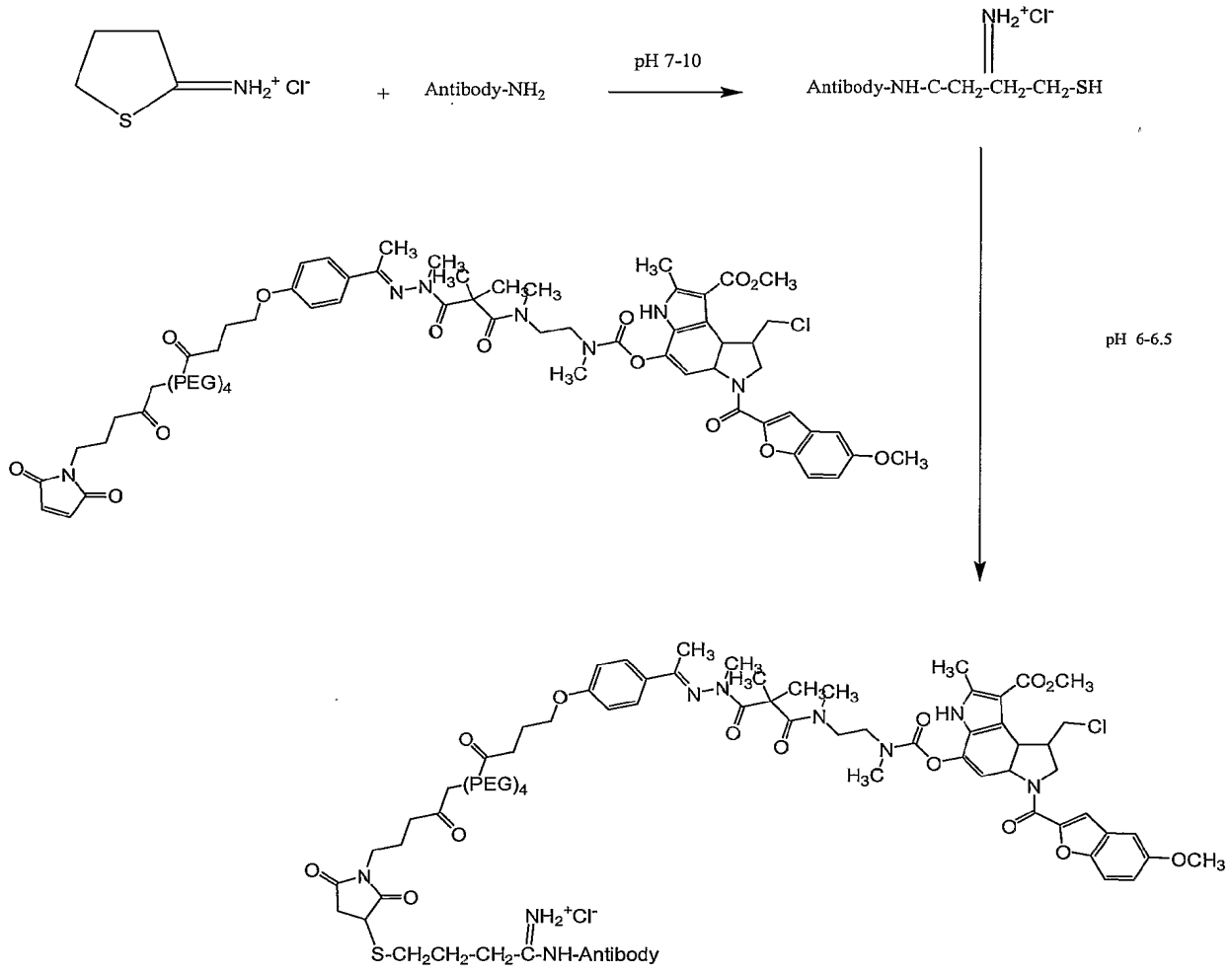
DMDA-2,2-Dimethylmalonic- Boc *N*'-methylhydrazine (**3**)

To a solution of Compound **2** (0.5 gm, 0.0011 moles) in MeOH (30 ml) was added 10% Pd/C (15 mg) and the reaction placed on a Parr hydrogenator for 30 minutes. The catalyst was filtered off and filtrate concentrated in vacuo to a clear oil to yield Compound **3** (0.38 gm). Product was confirmed by NMR (^1H, $CDCl_3$): δ 1.45 (s, 15H) 2.45 (s, 3H) 2.85 (s, 6H), 3.16 (s, 3H) 4.64 (m, 1H) 10.6 (bs, 1H); NMR (^{13}C, $CDCl_3$) δ 24.1, 28.57, 35.15, 35.58, 36.66, 47.01, 48.51, 81.11, 155.17, 173.56, 176.24

Synthesis of Compound **4**

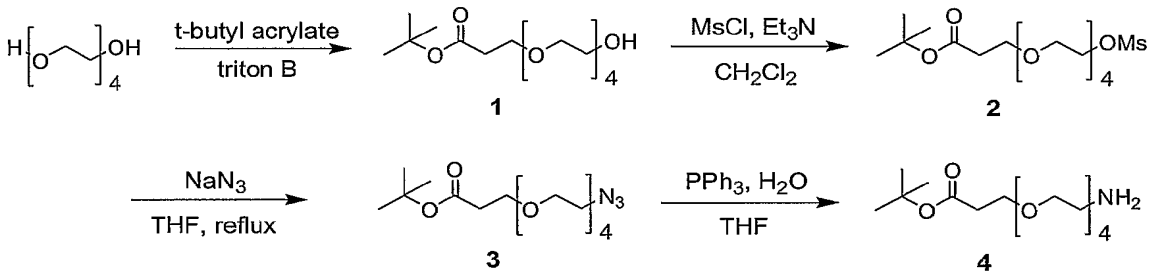
To a 15 ml RBF equipped with a stir bar, was combined Compound **3** (50 mg, 0.1513 mmoles), PNPC-1918 (20 mg, 0.0315 mmoles) and DCM (5 ml). The solution was stirred for 30 minutes, then triethylamine (25 *u*L, 0.1794 mmoles) was added and the bright yellow solution was stirred for 1 hr. The solution was concentrated in vacuo to a yellow oil and purified by column chromatography (100% DCM to 1:1 EtOAc/DCM) to yield Compound **4** as an off-white solid, 22 mg, (84%). Product was confirmed by mass spec: *m/z* 825.7 $[M + 1]^+$
HPLC retention time: 7.65 min (mass spec)

3.2 Synthesis of an antibody-drug conjugate having a 5-membered hydrazine linker

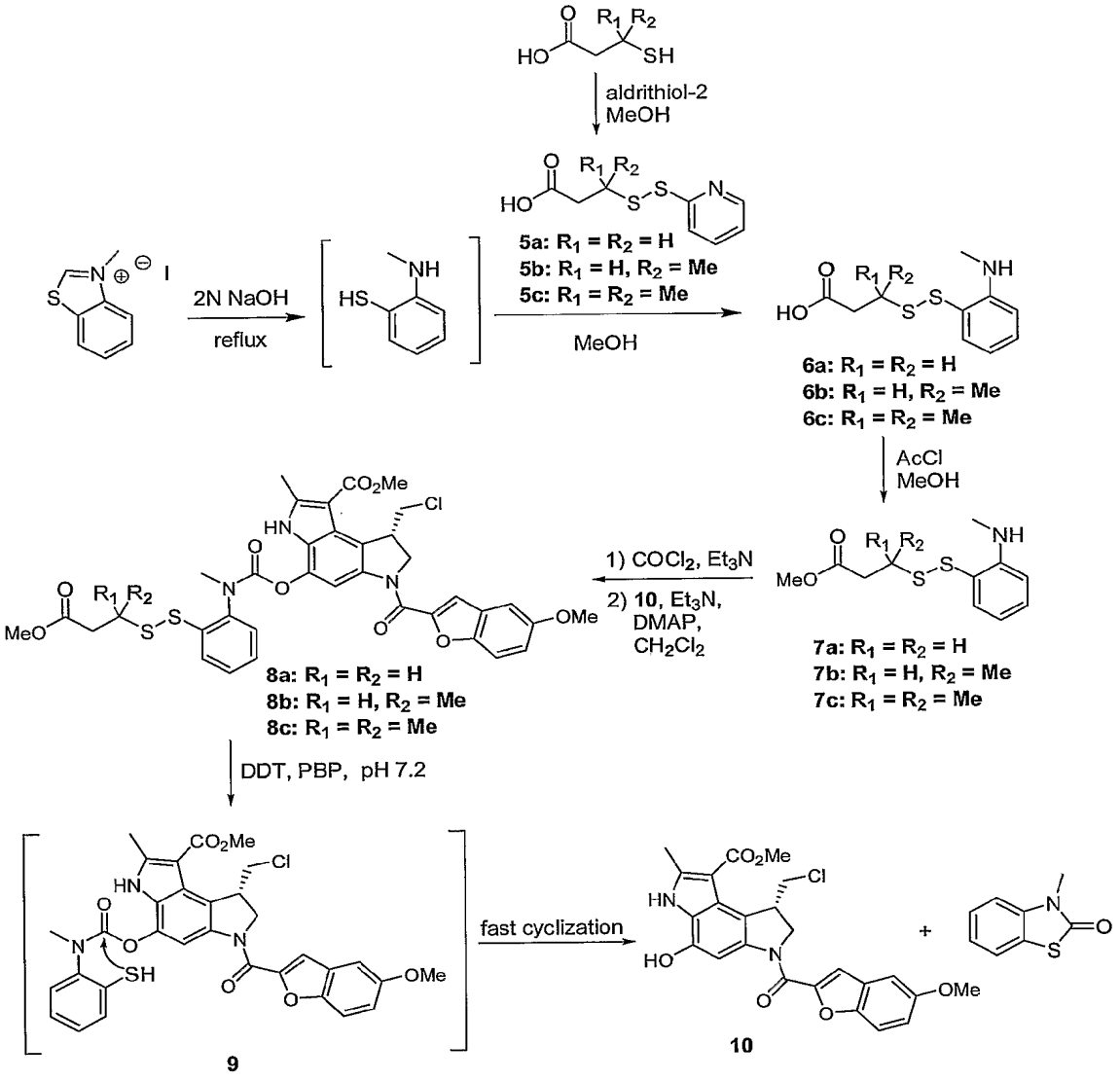


This scheme demonstrates the conjugation of an antibody to a linker-drug complex. These methodologies are well known in the pharmaceutical art. Examples of other reactive sites includes maleimides, haloacetamides with thiols on a ligand, thiols that react with disulfides on a ligand, hydrazides that react with aldehydes and ketones on a ligand, and hydroxysuccinimides, isocynates, isothiocyanates, and anhydride that react with amino group on a ligand.

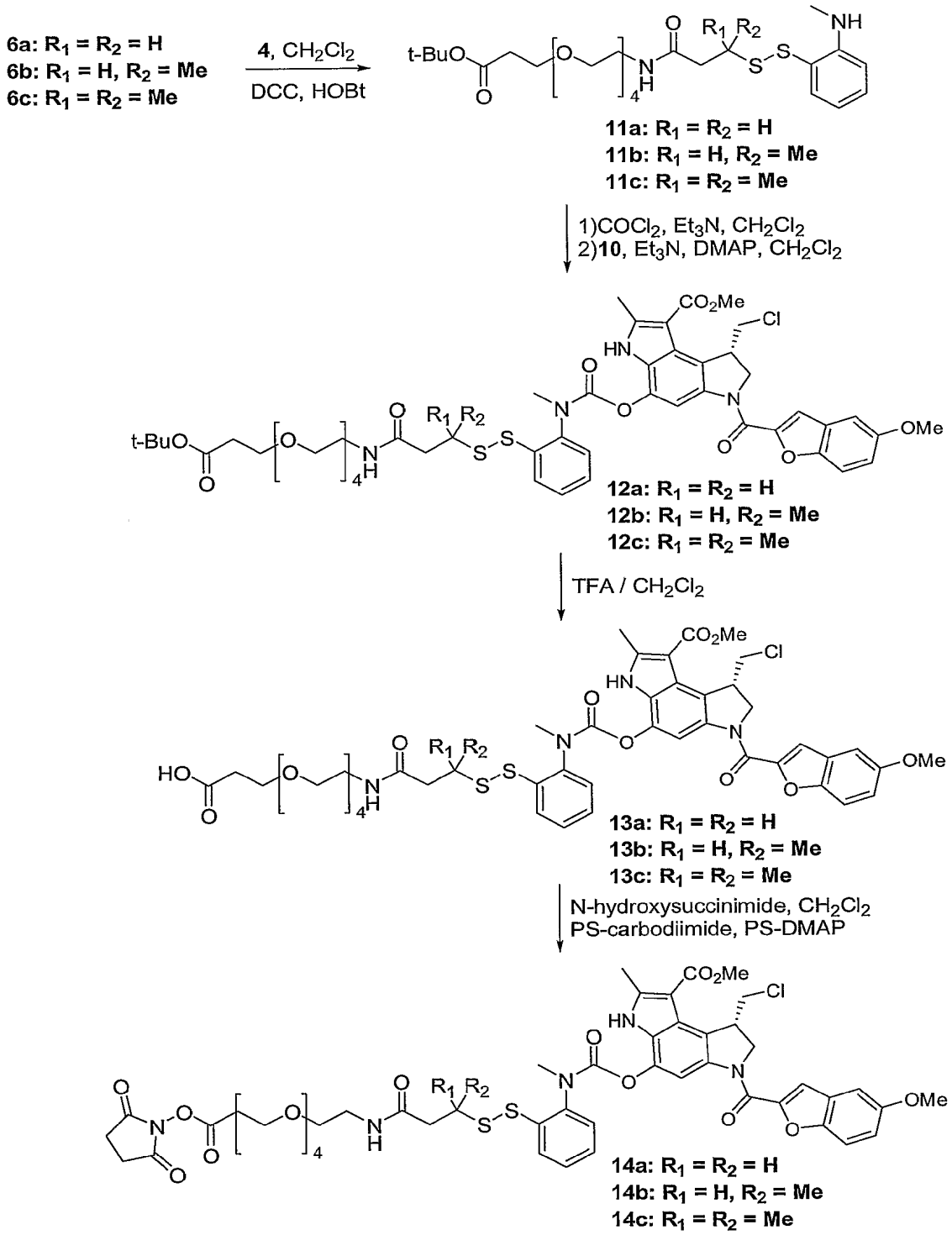
EXAMPLE 4: Synthesis of Disulfide Membered Linker Conjugates



Scheme 1


aldrithiol-2
MeOH
5a: R1 = R2 = H
5b: R1 = H, R2 = Me
5c: R1 = R2 = Me
2N NaOH
reflux
MeOH
6a: R1 = R2 = H
6b: R1 = H, R2 = Me
6c: R1 = R2 = Me
AcCl
MeOH
1) COCl2, Et3N
2) 10, Et3N,
DMAP,
CH2Cl2
7a: R1 = R2 = H
7b: R1 = H, R2 = Me
7c: R1 = R2 = Me
8a: R1 = R2 = H
8b: R1 = H, R2 = Me
8c: R1 = R2 = Me
DDT, PBP, pH 7.2
fast cyclization
9
10

Scheme 2


6a: R1 = R2 = H
6b: R1 = H, R2 = Me
6c: R1 = R2 = Me
4, CH2Cl2
DCC, HOBt
11a: R1 = R2 = H
11b: R1 = H, R2 = Me
11c: R1 = R2 = Me
1)COCl2, Et3N, CH2Cl2
2)10, Et3N, DMAP, CH2Cl2
12a: R1 = R2 = H
12b: R1 = H, R2 = Me
12c: R1 = R2 = Me
TFA / CH2Cl2
13a: R1 = R2 = H
13b: R1 = H, R2 = Me
13c: R1 = R2 = Me
N-hydroxysuccinimide, CH2Cl2
PS-carbodiimide, PS-DMAP
14a: R1 = R2 = H
14b: R1 = H, R2 = Me
14c: R1 = R2 = Me

Scheme 3

4.1a Synthesis of Compound **1**. To a flask containing PEG_4 (3.88 g, 20 mmole) was added triton B (40% solution in methanol, 1.08 mL, 0.25 mmole) and *tert*-butyl acrylate (3.62 mL, 24 mmole) followed after 15 min. The mixture was stirred at room temperature overnight. The mixture was concentrated *in vacuo* and the residue was purified by flash chromatography on silica gel with 1 % methanol in dichloromethane as eluent to give the title compound as an colorless oil (2.35 g, 36%). ^{1}H NMR δ 1.45 (s, 9H), 2.5 (t, 2H), 3.65 (m, 18H).

4.1b Synthesis of Compound **2**. To a solution of Compound **1** (1.17 g, 3.6 mmole) in dichloromethane (10 mL) were added triethylamine (532 μL, 4 mmole) and methanesulfonyl chloride (309 μL, 4 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 1 % methanol in dichloromethane as eluent to give the title compound as an yellow oil (1.3 g, 89%). ^{1}H NMR δ 1.43 (s, 9H), 2.48 (t, 2H), 3.07 (s, 3H), 3.62-3.70 (m, 14H), 3.76 (m, 2H), 4.37 (m, 2H).

4.1c Synthesis of Compound **3**. To a solution of Compound **2** (1.3 g, 3.25 mmole) in ethanol (10 mL) was added sodium azide (423 mg, 6.5 mmole). The mixture thus obtained was refluxed overnight. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 1 % methanol in dichloromethane as eluent to give the title compound as an yellow oil (1.01 g, 90%). ^{1}H NMR δ 1.45 (s, 9H), 2.50 (t, 2H), 3.40 (t, 2H), 3.62-3.73 (m, 16H).

4.1d Synthesis of Compound **4**. To a solution of Compound **3** (470 mg, 1.35 mmol) in ether (5 mL) containing H_2O (25 μL) was added triphenylphosphine (391 mg, 1.48 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 1 % methanol in dichloromethane as eluent to give the title compound as an yellow oil (325 mg, 75%). ^{1}H NMR δ 1.45 (s, 9H), 2.24 (bs, 2H), 2.51 (t, 2H), 2.91 (t, 2H), 3.56 (m, 2H), 3.63-3.66 (m, 12H). 3.72 (m, 2H).

4.1e Synthesis of Compound **5**. To a solution of 3-mercaptopropionic acid (1.22 g, 11.5 mmole) in methanol (10 mL) was added aldrithiol-2 (3.78 g, 17.25 mmole). The mixture thus obtained was stirred at room temperature for 3 hours. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 30 % ethyl acetate in hexanes as eluent to give the title compound as an oil (2.44 g, 98%). ^{1}H NMR δ 2.8 (t, 2H), 3.05 (t, 2H), 7.14 (m, 1H), 7.67 (m, 2H), 8.48 (m, 1H).

Compound **5b**: ^{1}H NMR δ 1.43 (d, 3H), 2.61 (m, 1H), 2.76 (m, 1H), 3.40 (m, 1H), 7.17 (m, 1H), 7.66 (m, 2H), 8.45 (m, 1H).

4.1f Synthesis of Compound **6**. 3-Methyl benzothiazolium iodide (1g, 3.6 mmole) was dissolved in 2 N sodium hydroxide aqueous solution (10 mL) and the mixture was stirred for 6 hours at 100 °C then acidified with 6 N hydrochloric acid aqueous solution to pH 4 and extracted with diethyl ether. The organic layer was dried over Na_2SO_4, rotary evaporated *in vacuo* and the residue was dissolved in methanol (10 mL) and compound 5a (776 mg, 3.6 m mole) was added. The mixture was stirred at room temperature for 1 hour. The mixture was concentrated to dryness and the residue was purified by flash chromatography on silica gel with 1% methanol in dichloromethane as eluent to give the title compound as a yellow oil (482 mg, 55%). ^{1}H NMR δ 2.85 (m, 2H), 2.95 (m, 5H), 6.64 (m, 2H), 7.3 (m, 1H), 7.4 (dd, 1H); MS (ES) 244 ($M+H^+$), 487 ($2M+H^+$).

Compound **6b**: ^{1}H NMR δ 1.35 (d, 3H), 2.48 (m, 1H), 2.92 (s, 3H), 3.02 (m, 1H), 3.34 (m, 1H), 6.62 (m, 2H), 7.28 (m, 1H), 7.44 (m, 1H) ; MS (ES) 258 ($M+H^+$).

Compound **6c**: ^{1}H NMR δ 1.45 (s, 6H), 2.70 (s, 2H), 2.93 (s, 3H), 6.62 (m, 2H), 7.24 (m, 1H), 7.51 (m, 1H) ; MS (ES) 272 ($M+H^+$), 294 ($M+Na^+$), 310 ($M+K^+$).

4.1g Synthesis of Compound **7**. To a solution of Compound **6a** (28 mg, 0.115 mmole) in anhydrous methanol (1 mL) was added acetyl chloride (13 μL, 0.173 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 10 % ethyl acetate in hexanes as eluent to give the title compound as an oil (24 mg, 83%). ^{1}H NMR δ 2.08 (m, 2H), 2.93 (s, 3H), 2.95

(m, 2H), 3.70 (s, 3H), 6.63 (m, 2H), 7.28 (m, 2H), 7.40 (m, 2H) ; MS (ES) 258 ($M+H^+$), 280 ($M+Na^+$), 296 ($M+K^+$).

Compound **7b**: ^{1}H NMR δ 1.32 (d, 3H), 2.45 (m, 1H), 2.92 (s, 3H), 2.93 (m, 1H), 3.35 (m, 1H), 3.67 (s, 3H), 6.62 (m, 2H), 7.26 (m, 1H), 7.44 (m, 1H) ; MS (ES) 272 ($M+H^+$).

Compound **7c**: ^{1}H NMR δ 1.42 (s, 6H), 2.66 (s, 2H), 2.93 (s, 3H), 3.62 (s, 3H), 6.62 (m, 2H), 7.24 (m, 1H), 7.51 (m, 1H) ; MS (ES) 286 ($M+H^+$), 308 ($M+Na^+$), 324 ($M+K^+$).

4.1h Synthesis of Compound **8**. To a solution of Compound **7a** (24 mg, 0.093 mmole) in dichloromethane (1 mL) were added triphosgene (28 mg, 0.093 mmole) and triethylamine (37 μL, 0.28 mmole) at 0°C. The mixture was stirred for 1 hour. The mixture was concentrated to dryness and the residue was used in next step without further purification.

The crude material was dissolved in dichloromethane (1 mL) and the Compound **8a** (35 mg, 0.074 mmole), and DMAP (23 mg, 0.190 mmole) were added. The mixture thus obtained was stirred at room temperature for overnight. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 1 % methanol in dichloromethane as eluent to give the title compound as an yellow oil (53 mg, 76%). ^{1}H NMR δ 2.70 (s, 3H), 2.74 (m, 2H), 3.06 (m, 2H), 3.34 (m, 1H), 3.35 and 3.36 (2s, 3H), 3.63 and 3.64 (2s, 3H), 3.86 (m, 1H), 3.88 (s, 3H), 3.93 and 3.94 (2s, 3H), 4.48 (m, 1H), 4.55 (m, 1H), 4.79 (m, 1H), 7.05 (m, 1H), 7.11 (m, 1H), 7.26-7.52 (m, 5H), 7.85 (d, 1H), 8.1 (bs, 1H), 8.98 and 9.08 (2s, 1H) ; MS (ES) 753 ($M+H^+$).

Compound **8b**: ^{1}H NMR δ 1.38 (m, 3H), 2.52 (m, 1H), 2.69 (m, 3H), 2.79 (m, 1H), 3.33 (m, 1H), 3.37 (2s, 3H), 3.64 (m, 3H), 3.88 (s, 3H), 3.84-3.90 (m, 1H), 3.93 (2s, 3H), 4.48 (m, 1H), 4.57 (m, 1H), 4.78 (m,1H), 7.06 (m, 1H), 7.12 (m, 1H), 7.26-7.43 (m, 3H), 7.50 (m, 2H), 7.86 (m, 1H), 8.1 (bs, 1H), 8.99, 9.08, 9.13 and 9.22 (4s, 1H) ; MS (ES) 767 ($M+H^+$).

Compound **8c**: ^{1}H NMR δ 1.44 (m, 6H), 2.63 (d, 2H), 2.70 (s, 3H), 3.35 (m, 1H), 3.38 and 3.39 (2s, 3H), 3.63 and 3.64 (2s, 3H), 3.87 (m, 1H), 3.88 (s, 3H), 3.93 and 3.94 (2s, 3H), 4.48 (m, 1H), 4.55 (m, 1H), 4.79 (m, 1H), 7.05 (m, 1H), 7.12 (m, 1H), 7.31-7.39 (m, 3H), 7.49 (m, 2H), 7.89 (d, 1H), 8.1 (bs, 1H), 9.12 and 9.23 (2s, 1H) ; MS (ES) 781 ($M+H^+$).

4.1i Synthesis of Compounds **9** and **10**. To a solution of Compound **8a** (0.1 mg) in PBS buffer solution (pH 7.2) / methanol (300 µL, 2/1) was added a 20 mM solution of DTT (100 µL, 15 equiv.) and monitored the progress of the reaction by HPLC. The reaction underwent too fast to detect, after few seconds the reaction was completed already to give product Compound **10** quantitatively. The reaction intermediate Compound **9** was not detected.

4.1j Synthesis of Compound **11**. To a solution of Compound **6a** (66 mg, 0.2 m mole) in dichloromethane (1 mL) were added DCC (47 mg, 0.22 m mole), HOBt (31 mg, 0.22 mmole) and the compound 4 (50 mg, 0.2 m mole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified by flash chromatography on silica gel with 1 % methanol in dichloromethane as eluent to give the title compound as an yellow oil (70 mg, 62%). ^{1}H NMR δ 1.44 (s, 9H), 2.51 (t, 1H), 2.63 (t, 2H), 2.93 (d, 3H), 3.01 (t, 2H), 3.45 (m, 2H), 3.55 (m, 2H), 3.64 (m, 12H), 3.71 (t, 2H), 5.01 (bs, 1H), 6.38 (bt, 1H), 6.62 (m, 2H), 7.27 (m, 1H), 7.43 (dd, 1H). MS (ES) 491 (M-56+H$^+$), 513 (M-56+Na$^+$), 547 (M+H$^+$), 569 (M+Na$^+$)

Compound **11b**: ^{1}H NMR δ 1.34 (d, 3H), 1.45 (s, 9H), 2.30 (m, 1H), 2.5 (t, 2H), 2.69 (m, 1H), 2.93 (d, 3H), 3.37-3.55 (m, 5H), 3.63 (m, 12H), 3.71 (t, 2H), 4.99 (bs, 1H), 6.13 (bt, 1H), 6.62 (m, 2H), 7.25 (m, 1H), 7.48 (dd, 1H). MS (ES) 505 (M-56+H$^+$), 527 (M-56+Na$^+$), 543 (M-56+K$^+$), 561 (M+H$^+$), 583 (M+Na$^+$).

Compound **11c**: 1.43 (s, 3H), 1.45 (s, 9H), 2.46 (s, 2H), 2.5 (t, 2H), 2.92 and 2.94 (2s, 3H), 3.33 (m, 2H), 3.47 (t, 2H), 3.63 (m, 12H), 3.70 (t, 2H), 6.06 (bt, 1H), 6.63 (m, 2H), 7.25 (m, 1H), 7.54 (d, 1H) ; MS (ES) 519 (M-56+H$^+$), 541 (M-56+Na$^+$), 575 (M+H$^+$), 597 (M+Na$^+$).

4.1k Synthesis of Compound **12**: To a suspension of Compound **11a** (20 mg, 0.037 mmole) in dichloromethane (1 mL) were added triethylamine (15 µL, 0.11 mmole) and a solution of 2 N phosgene in toluene (55 µL, 0.11 m mole) at 0 °C. The mixture was stirred at room temperature for 1 hour. The mixture was concentrated and the residue was dissolved in dichloromethane (1 mL) and the compound 10 (14 mg, 0.030 mmole) and DMAP (9 mg, 0.076 m mole) were added. The mixture thus obtained was stirred at room temperature overnight. The solvent was

evaporated and the residue was purified by flash chromatography on silica gel with 1 % methanol in dichloromethane as eluent to give the title compound as an yellow oil (23 mg, 74%). ^{1}H NMR δ 1.44 (s, 9H), 2.49 (t, 2H), 2.67 (m, 2H), 2.65 and 2.67 (2s, 3H), 3.07 (m, 2H), 3.33 (s, 3H), 3.40 (m, 3H), 3.51 (m, 2H), 3.60 (m, 12H), 3.69 (m, 2H), 3.87 (s, 3H), 3.92 (s, 3H), 3.93 (m, 1H), 4.52 (m, 2H), 4.78 (m, 1H), 6.65, 6.74 and 6.97 (3bt, 1H), 7.06 (d, 1H), 7.12 (s, 1H), 7.29-7.42 (m, 3H), 7.50 (m, 2H), 7.87 (d, 1H), 8.10 and 8.15 (2bs, 1H), 9.79 and 9.58 (2s, 1H) ; MS (ES) 986 ($M+H^+$-56), 1042 ($M+H^+$).

Compound **12b**: ^{1}H NMR δ 1.32 (m, 3H), 1.44 (s, 9H), 2.39 (m, 1H), 2.48 (m, 2H), 2.60 (m, 1H), 2.67 and 2.69 (2s, 3H), 3.32 and 3.35 (2s, 3H), 3.38-3.72 (m, 20H), 3.88 (s, 3H), 3.93 (s, 3H), 3.94 (m, 1H), 4.52 (m, 2H), 4.77 (m, 1H), 6.53, 6.67 and 6.72 (3bt, 1H), 7.06 (d, 1H), 7.12 (s, 1H), 7.29-7.39 (m, 3H), 7.49 (m, 2H), 7.88 (d, 1H), 8.12 and 8.25 (2bs, 1H), 9.13, 9.36, 10.08 and 10.21 (4s, 1H) ; MS (ES) 1000 ($M+H^+$-56), 1056 ($M+H^+$), 1078 ($M+Na^+$), 1084 ($M+K^+$).

Compound **12c**: ^{1}H NMR δ 1.30-1.42 (m, 3H), 1.44 (s, 9H), 2.45-2.52 (m, 4H), 2.69 and 2.72 (2s, 3H), 3.34 and 3.35 (2s, 3H), 3.39-3.72 (m, 19H), 3.88 (s, 3H), 3.925 and 3.93 (2s, 3H), 3.94 (m, 1H), 4.53 (m, 2H), 4.80 (m, 1H), 6.63 (m, 1H), 7.06 (dd, 1H), 7.13 (d, 1H), 7.25-7.39 (m, 3H), 7.50 (m, 2H), 7.89 (d, 1H), 8.10 and 8.27 (2bs, 1H), 9.99 and 10.191 (2s, 1H); MS (ES) 1014 ($M+H^+$-56), 1070 ($M+H^+$), 1108 ($M+K^+$).

4.11 Synthesis of Compound **13**. Compound **12a** (23 mg, 0.022 mmole) was dissolved in the solution of trifluoroacetic acid and dichloromethane (1 mL, 1 / 1) and the mixture was stirred at room temperature for 30 min and concentrated to give the product (21 mg, 100%) ^{1}H NMR δ 2.60 (t, 2H), 2.67 and 2.68 (2s, 3H), 2.75 (m, 2H), 3.07 (m, 2H), 3.34 (s, 3H), 3.38-3.64 (m, 21H), 3.76 (t, 2H), 3.88 (s, 3H), 3.92 (s, 3H), 3.93 (m, 1H), 4.53 (m, 2H), 4.78 (m, 1H), 7.06 (d, 1H), 7.13 (s, 1H), 7.31-7.43 (m, 3H), 7.49 (m, 2H), 7.87 (d, 1H), 8.10 and 8.15 (2bs, 1H), 9.44 and 9.65 (2s, 1H) ; MS (ES) 986 ($M+H^+$), 1008 ($M+Na^+$), 1024 ($M+K^+$).

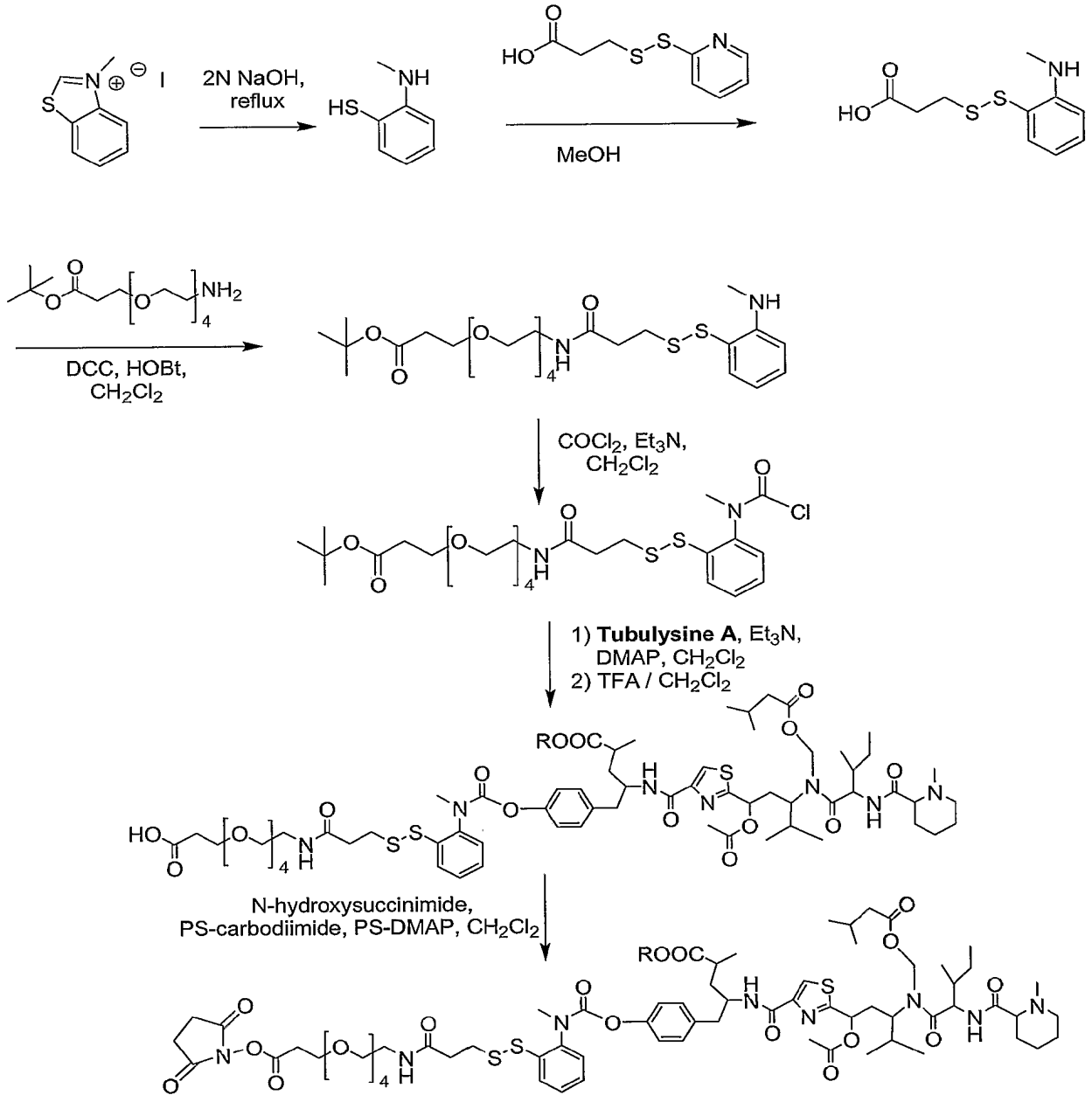
Compound **13b**: ^{1}H NMR δ 1.34 (m, 3H), 2.56 (m, 1H), 2.62 (m, 2H), 2.68 (m, 3H), 2.8 (m, 1H), 3.35-3.36 (2s, 3H), 3.40-3.70 (m, 18H), 3.77 (t, 2H), 3.88 (s, 3H), 3.93 and 3.95 (2s, 3H), 3.94 (m, 1H), 4.54 (m, 2H), 4.79 (m, 1H), 7.07 (d, 2H), 7.13 (s, 1H), 7.30-7.42 (m,

3H), 7.49 (m, 2H), 7.88 (d, 1H), 8.11 and 8.25 (2bs, 1H), 9.22, 9.37,9.80 and 9.92 (4s, 1H) ; MS (ES) 1000 ($M+H^+$), 1022 ($M+Na^+$), 1038 ($M+K^+$).

Compound **13c**: ^1H NMR δ 1.30-1.45 (m, 6H), 2.54 (m, 2H), 2.61 (m, 2H), 2.68 and 2.69 (2s, 3H), 3.35-3.36 (2s, 3H), 3.40-3.70 (m, 17H), 3.77 (t, 2H), 3.88 (s, 3H), 3.92 and 3.93 (2s, 3H), 3.94 (m, 1H), 4.50 (m, 2H), 4.80 (m, 1H), 7.08 (m, 2H), 7.12 (d, 1H), 7.29-7.39 (m, 3H), 7.49 (m, 2H), 7.89 (m, 1H), 8.10 and 8.25 (2bs, 1H), 9.88 and 10.04 (2s, 1H) ; MS (ES) 1014 ($M+H^+$), 1036 ($M+Na^+$), 1054 ($M+K^+$).

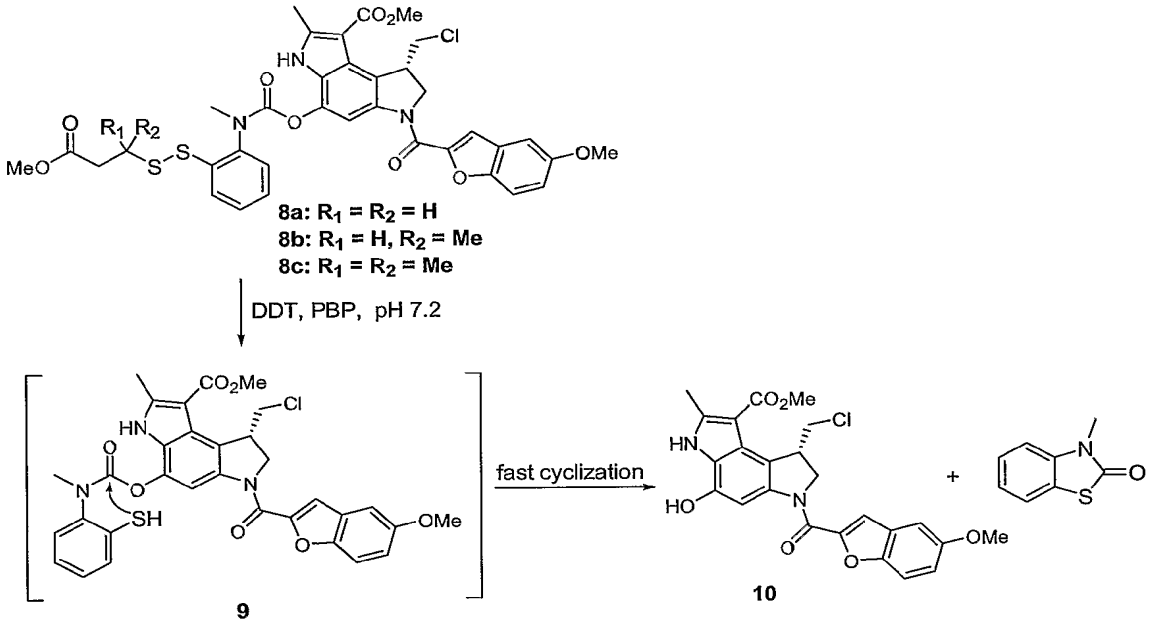
4.1m Synthesis of Compound **14a**. To a solution of Compound **13a** (5.4 mg, 0.0054 mmole) in dichloromethane (1 mL) were added PS-carbodiimide (11.5 mg, 0.94 mmole / g, 0.0108 mmole), and PS-DMAP (7.2 mg, 1.49 m mole / g, 0.0108 m mole). The mixture thus obtained was stirred at room temperature overnight, filtrated and concentrated to give the product. MS (ES) 1082 ($M+H^+$).

4.2 Synthesis of Disulfide Linker Conjugated with Tubulysin A



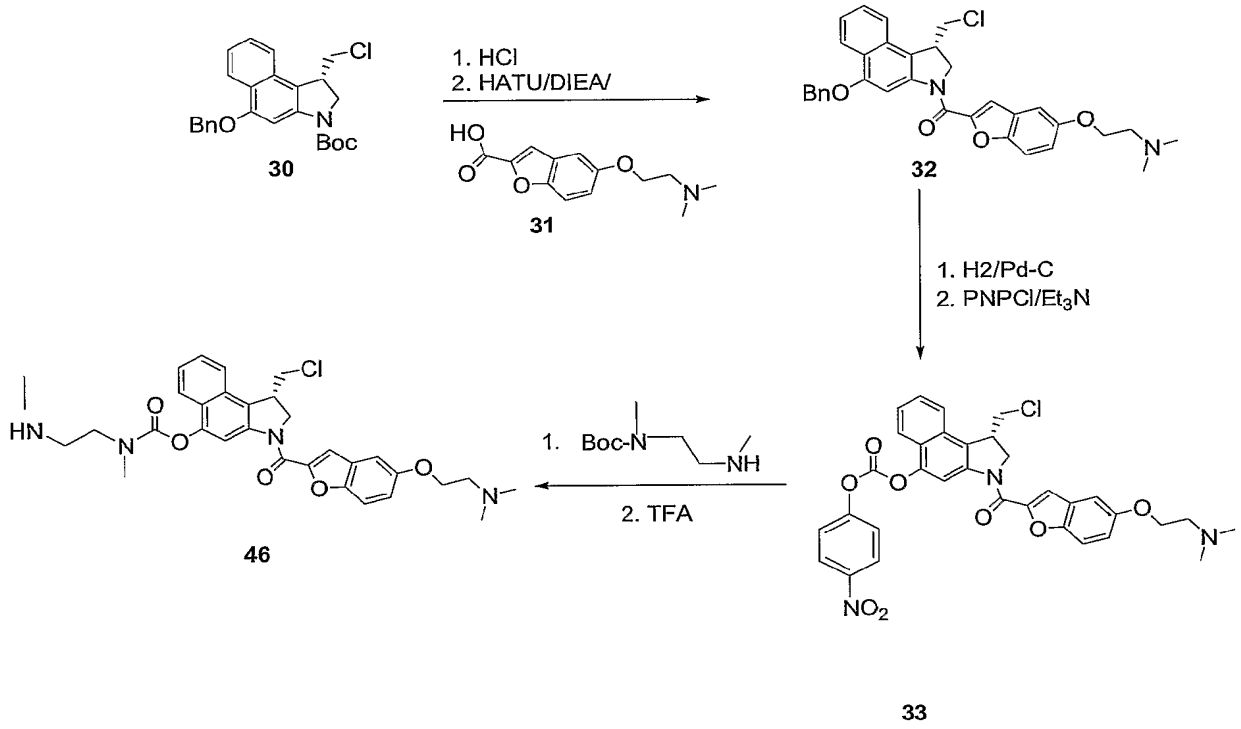
The drug Tubulysin A can be conjugated to the disulfide linker of the current invention using the mechanism shown hereinabove. Other drugs and other linkers of the current invention can be synthesized using similar reaction schemes.

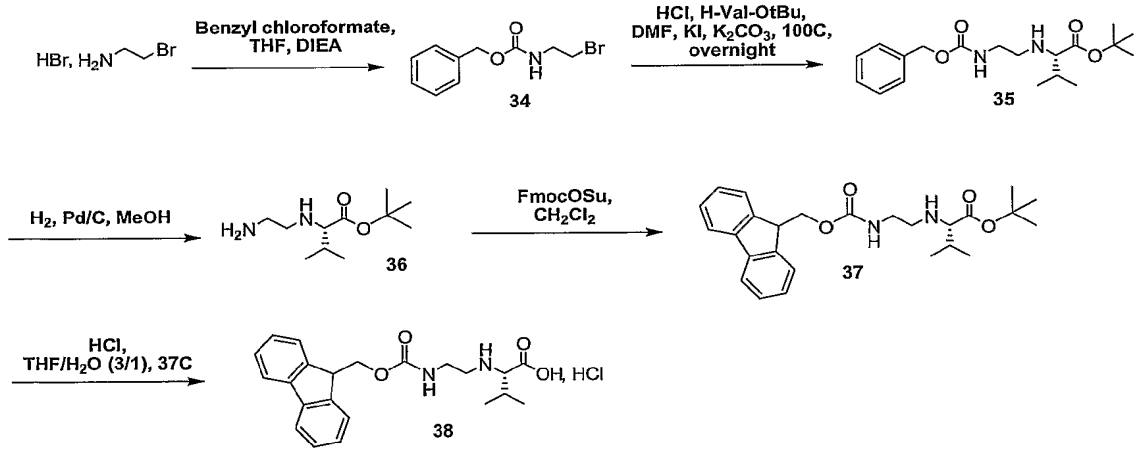
4.3 Rate of Cyclization of a Disulfide Linker

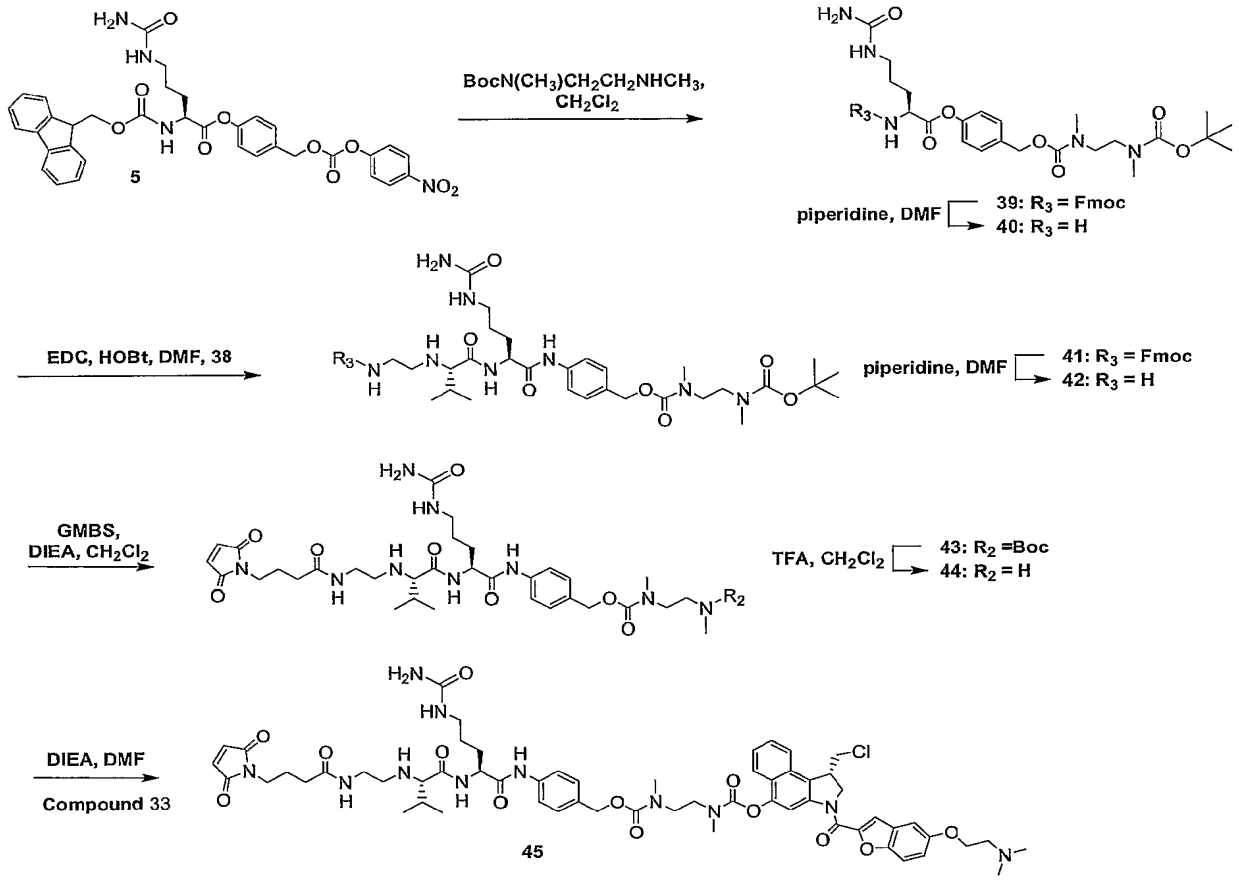


To a solution of Compound **8a** (0.1 mg) in PBS buffer solution (pH 7.2) / methanol (300 μL, 2/1) was added a 20 mM solution of DTT (100 μL, 15 equiv.) and the progress of the reaction was monitored by HPLC. The reaction underwent rapid cyclization, with the reaction being completed within a few seconds to give product 10 quantitatively. The reaction intermediate 9 was not detected.

Example 5


1. HCl
2. HATU/DIEA/
30
31
32
1. H2/Pd-C
2. PNPCl/Et3N
1. Boc-N
NH
2. TFA
46
33


HBr, H2N
Br
Benzyl chloroformate,
THF, DIEA
34
HCl, H-Val-OtBu,
DMF, KI, K2CO3, 100C,
overnight
35
H2, Pd/C, MeOH
36
FmocOSu,
CH2Cl2
37
HCl,
THF/H2O (3/1), 37C
OH, HCl
38



Synthesis of Compound **32**. To a solution of Compound **30** (120 mg, 0.28 mmole) in ethyl acetate (10 mL) was bubbled HCl gas for 5 min. The reaction mixture was stirred at RT for another 30 min and then the mixture was concentrated. Ether was added to the reaction mixture and the white precipitate was collected on a filter funnel. Solid was dried overnight under vacuum to give 100mg of the desired product which was confirmed by LC-MS (ESI) 324 ($M+H^+$) and used in next step without further purification. To a solution of this compound (100 mg, 0.24 mmole) in DMF (5 mL) were added compound **31** (65 mg, 0.26 mmole), HATU (100 mg, 0.26 mmole) and TEA (91 uL, 0.52 mmole). The mixture thus obtained was stirred at room temperature for 3 hrs. The solvent was evaporated and the residue was purified on semi-preparative HPLC with 0.1% TFA in water and acetonitrile as eluent to give compound **32** as an oil (110 mg, 80%). The desired product was confirmed by LC-MS (ESI) 555 ($M+H^+$).

Synthesis of Compound **33**. A solution of Compound **32** (110 mg, 0.2 mmole) and palladium on charcoal (20 mg) in DCM (10 mL) and methanol (5 mL) was stirred under hydrogen atmospheric pressure at room temperature for 12 hrs. The palladium was filtrated and the reaction mixture was concentrated and the residue was purified on semi-preparative HPLC with 0.1% TFA in water and acetonitrile as eluent to give the desired compound as an oil (80 mg, 78%) LC-MS (ESI) 465 ($M+H^+$). To a solution of the residue (80 mg, 0.17 mmole) in dichloromethane (10 mL) and THF (5 mL) was added PNPCl (4-nitrophenyl chloroformate) (137 mg, 0.68 mmole) and triethyl amine (144 uL, 1.02 mmol) at 0°C. The mixture thus obtained was stirred for 30 min at 0°C and then at room temperature for 12 hrs. The reactiom mixture was concentrated under vaccum, and the residue was precipitated using ethyl ether (100 mL) to give compound **33** as a yellow solid (90 mg, 82%) which was dried under vaccum and confirmed by LC-MS (ESI) 631 ($M+H^+$).

Synthesis of Compound 46: To a solution of compound 33 (60 mg, 0.1 mmole) in dichloromethane (10 mL) was added Boc-N,N dimethyl entyl diamaine (84 mg, 0.38 mmole) and triethyl amine (26 uL, 0.1 mmol) at room temperature. The mixture thus obtained was stirred at room temperature for 12 hrs. The reactiom mixture was concentrated under vaccum, and the residue was precipitated using ethyl ether (100 mL) to give Boc protected compound 34 which was used for the next step without further purification. Boc protected compound 34 was dissolved in 10 mL of TFA and the reaction mixtured was stirred at room temperature for 60 min. The reactiom mixture was concentrated under vaccum, and the residue was precipitated using ethyl ether (100 mL) to give compound **46** as a yellow solid which was dried under vaccum and confirmed by LC-MS (ESI) 631 ($M+H^+$).

Synthesis of Compound 34: To a solution of 2-bromoethylamine bromide (5 g, 24.4 mmole) in DMF (50 mL) was added diisopropylethylamine (8.5 mL, 48.8 mmole) and benzyl chloroformate (3.48 mL, 24.4 mmole). The mixture thus obtained was stirred at room temperature for 2 hours. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with ethyl acetate/hexanes (3/7) as eluent to give the desired

compound **34** as an oil (4 g, 64%). ^{1}H NMR ($CDCl_3$) δ 3.54 (bs, 2H), 3.61 (bs, 2H), 5.12 (s, 2H), 7.36 (m, 5H).

Synthesis of Compound 35: To a solution of Compound **34** (3.34 g, 12.99 mmole) and valine tert-butyl ester (3.27 g, 15.59 mmole) in DMF (50 mL) was added potassium carbonate (5.39 g, 38.97 mmole) and potassium iodide (2.59 g, 15.59 mmole). The mixture thus obtained was stirred at 100°C overnight. The reaction mixture was concentrated and the residue was purified by flash chromatography on silica gel with ethyl acetate/hexanes (2/8) as eluent to give the desired compound **35** as an oil (3.12 g, 69%). ^{1}H NMR ($CDCl_3$) δ 0.92 (m, 6H), 1.46 (s, 9H), 1.86 (m, 1H), 2.53 (m, 1H), 2.80 (m, 2H), 3.18 (m, 1H), 3.31 (m, 1H), 5.10 (s, 2H), 5.25 (bs, 1H), 7.36 (m, 5H); LC-MS (ESI) 296 (M+H-*t*butyl$^+$), 352 (M+H$^+$).

Synthesis of Compound 36. A solution of Compound **35** (3.4 g, 9.72 mmole) and palladium on charcoal (200 mg) in methanol (30 mL) was placed under hydrogen atmospheric pressure at room temperature. The mixture thus obtained was stirred at room temperature for 2 hours. The palladium was filtrated and the reaction mixture was concentrated to dryness to give the desired compound **36** as an oil (2.1 g, 98%)

Synthesis of Compound 37. To a solution of Compound **36** (2.1 g, 9.72 mmole) in dichloromethane (30 mL) was added FmocOSu (9-fluorenylmethoxycarbonyl-*N*-hydroxysuccinimide ester) (3.28 g, 9.72 mmole) at 0°C. The mixture thus obtained was stirred for 2 hours at 0°C. The solvent were removed on the rotovap, and the residue was purified by flash chromatography on silica gel with dichloromethane, followed by 0.5% methanol in dichloromethane and finally 1% methanol in dichloromethane as eluent to give the desired compound **37** as colorless oil (2.55 g, 60%). ^{1}H-NMR ($CDCl_3$) δ 0.95 (ft, 6H), 1.48 (s, 9H), 1.90 (m, 1H), 2.55 (m, 1H), 2.82 (m , 2H), 3.18 (m, 1H), 3.32 (m, 1H), 4.24 (m, 1H), 4.37 (m, 2H), 5.40 (bs, 1H), 7.30 (m, 2H), 7.39 (m, 2H), 7.60 (d, 2H), 7.75 (d, 2H) ppm; LC-MS (ESI) 383 (M+H-*t*butyl$^+$), 440 (M+H$^+$), 462 (M+Na$^+$), 478 (M+K$^+$).

Synthesis of Compound 38. To a solution of Compound **37** (177 mg, 0.4 mmole) in tetrahydrofuran-water (3/1, 8 mL) was bubbled HCl gas for 5 min. The reaction mixture was stirred at 37°C overnight then the mixture was concentrated to dryness to give the desired compound **38** as solid (168 mg, 98%) which was confirmed by LC-MS (ESI) 383 ($M+H^+$), 405 ($M+Na^+$) and used in next step without further purification. LC-MS (ESI) 383 ($M+H^+$), 405 ($M+Na^+$).

Synthesis of Compound 39. To a solution of Compound **5** (525 mg, 0.79 mmole) in DMF (5 mL) was added *N*-Boc-*N,N'*-dimethylethylenediamine (177 mg, 0.94 mmole). The mixture thus obtained was stirred at room temperature for 30 min. The solvent was removed and the residue was purified by flash chromatography on silica gel with dichloromethane, followed by 2% methanol in dichloromethane and finally 5% methanol in dichloromethane as eluent to give the desired compound **39** as colorless oil (364 mg, 65%). ^{1}H-NMR (CD_3OD) δ 1.39 (s, 9H), 1.56 (m, 2H), 1.70 (m, 1H), 1.82 (m , 1H), 2.70 and 2.82 (2s, 3H), 2.90 (s, 3H), 3.09 (m, 1H), 3.17 (m, 1H), 3.30 to 3.37 (m, 4H), 4.16 (t, 1H), 4.27 (m, 1H), 4.33 (d, 2H), 5.02 (bs, 2H), 7.24 to 7.36 (m, 6H), 7.51 to 7.65 (m, 4H), 7.74 (d, 2H) ppm; LC-MS (ESI) 618 ($M+H-Boc^+$), 662 ($M+H-t$butyl$^+$), 718 ($M+H^+$), 740 ($M+Na^+$), 1435 ($2M+H^+$).

Synthesis of Compound 40. Compound **40** was prepared as described above for Compound **17a** in 98% yield. LC-MS (ESI) 396 ($M+H-Boc^+$), 496 ($M+H^+$), 517 ($M+Na^+$), 533 ($M+K^+$), 992 ($2M+H^+$).

Synthesis of Compound 41. To a solution of Compound **40** (138 mg, 0.28 mmole) in DMF (4 mL) were added the Compound **38** (110 mg, 0.28 mmole), HOBt (36 mg, 0.28 mmole) and EDC (1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (50 mg, 0.28 mmole). The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified on semi-preparative HPLC with 0.1% TFA in water and acetonitrile as eluent to give the desired compound **41** as an oil (178 mg, 70%). ^{1}H-NMR (CD_3OD) δ 1.04 and 1.11 (2d, 6H), 1.40 (s, 9H), 1.58 (m, 2H), 1.77 (m, 1H), 1.88 (m , 1H), 2.24 (m, 1H), 2.72 and 2.84 (2s, 3H), 2.92 (s, 3H), 3.10 to 3.18 (m, 4H), 3.35 to 3.46 (m, 6H), 3.82 (d, 1H), 4.22 (t,

1H), 4.41 (m, 2H), 4.59 (m, 1H), 5.04 (bs, 2H), 7.28 to 7.40 (m, 6H), 7.55 (m, 2H), 7.63 (m, 2H), 7.78 (d, 2H) ppm; LC-MS (ESI) 760 ($M+H-Boc^+$), 804 ($M+H-t butyl^+$), 860 ($M+H^+$), 882 ($M+Na^+$), 899 ($M+K^+$).

Synthesis of Compound 42. Compound **42** was prepared as described above for Compound **17a** in 98% yield. LC-MS (ESI) 538 ($M+H-Boc^+$), 582 ($M+H-t butyl^+$), 638 ($M+H^+$), 660 ($M+Na^+$).

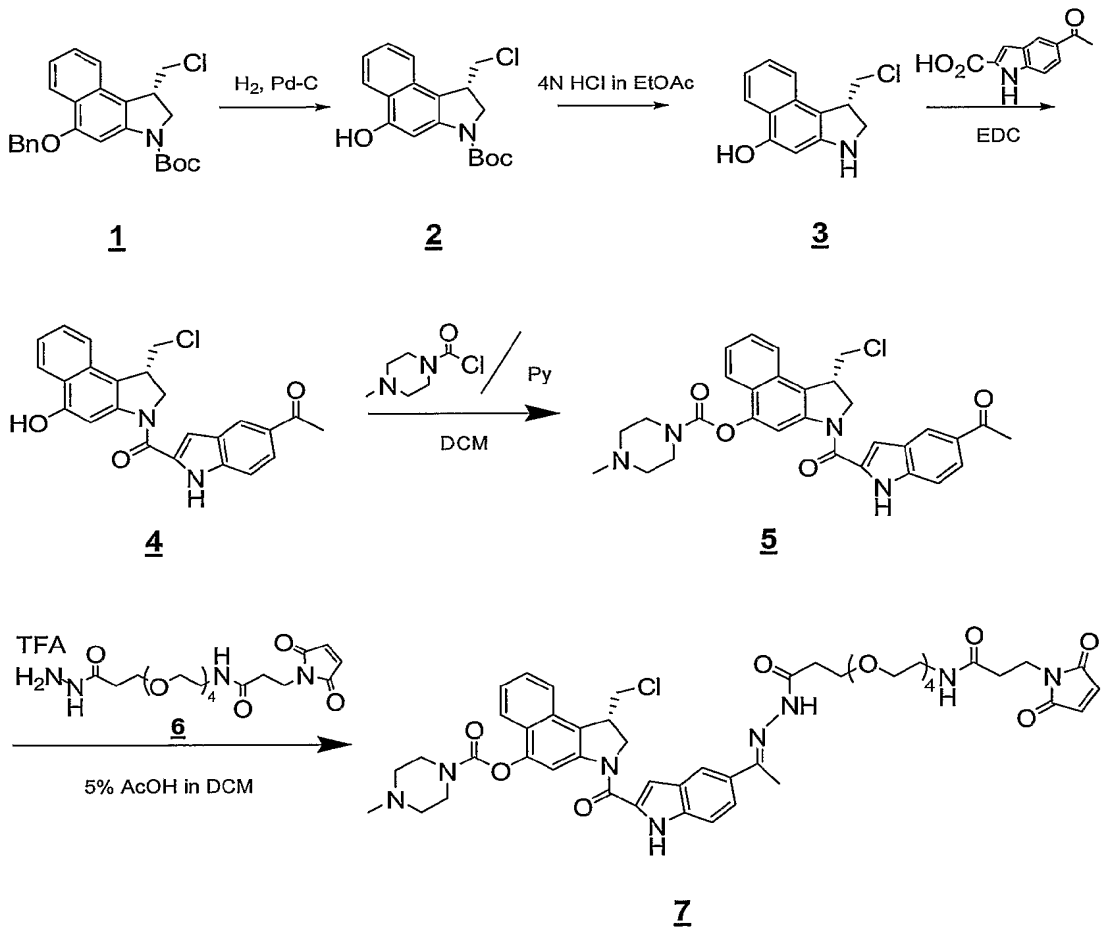
Synthesis of Compound 43. To a solution of Compound **42** (23 mg, 0.036 mmole) in dichloromethane (1 mL) were added GMBS (N-(maleimidobutyryloxy)succinimide ester) (14 mg, 0.05 mmole) and diisopropylethylamine (8.4 μL, 0.05 mmole) at 0°C. The mixture was warmed up to room temperature slowly and the stirring was continued for additional 30 min. The solvent was evaporated and the residue was purified on semi-preparative HPLC with 0.1% TFA in water and acetonitrile as eluent to give the desired compound **43** as an oil (26 mg, 79%). ^{1}H-NMR (CD_3OD) δ 1.06 and 1.12 (2d, 6H), 1.41 (s, 9H), 1.59 (m, 2H), 1.78 (m, 1H), 1.86 to 1.93 (m , 3H), 2.24 (m, 3H), 2.74 and 2.84 (2s, 3H), 2.93 (bs, 3H), 3.13 to 3.22 (m, 4H), 3.40 to 3.60 (m, 8H), 3.82 (d, 1H), 4.60 (m, 1H), 5.05 (bs, 2H), 6.80 (s, 2H), 7.32 (m, 2H), 7.57 (d, 2H), 8.78 (d, 1H) ppm; LC-MS (ESI) 703 ($M+H-Boc^+$), 747 ($M+H-t butyl^+$), 803 ($M+H^+$), 825 ($M+Na^+$), 841 ($M+K^+$).

Synthesis of Compound 44. Compound **44** was prepared as described above for Compound **15a** in 98% yield. LC-MS (ESI) 703 ($M+H^+$), 725 ($M+Na^+$).

Synthesis of Compound 45. To a solution of Compound **44** (15 mg, 0.016 mmole) and Compound **33** (10 mg, 0.016 mmole) in DMF (0.8 mL) was added diisopropylethylamine (5.5 μL, 0.032 mmole) at room temperature. The mixture thus obtained was stirred at room temperature overnight. The solvent was evaporated and the residue was purified on semi-preparative HPLC with 0.1% TFA in water and acetonitrile as eluent to give the desired compound **45** as an oil (10 mg, 45%). ^{1}H-NMR (CD_3OD) δ 1.02 to 1.13 (m, 6H), 1.55 (m, 2H), 1.74 (m, 1H), 1.84 to 1.92 (m , 3H), 2.20 to 2.27 (m, 3H), 2.95 to 3.14 (m, 16H), 3.47 to 3.84 (m, 12H), 3.98 (m, 1H), 4.2 to 4.34 (m, 3H), 4.57 (m, 1H), 4.69 (m, 2H), 5.07 to 5.17 (m, 2H), 6.78

(s, 2H), 7.16 to 7.23 (m, 3H), 7.30 (m, 1H), 7.38 to 7.47 (m, 3H), 7.52 to 7.58 (m, 3H), 7.81 to 7.92 (m, 2H), 8.25 (bs, 1H) ppm; LC-MS (ESI) 1194 ($M+H^+$), 1215 ($M+Na^+$), 1233 ($M+K^+$).

Example 6



Synthesis of Compound (2). A solution of **1** (100 mg, 0.24 mmol) and 10% Pd-C (35 mg) in $MeOH/CH_2Cl_2$ (1/2, 10 ml) was degassed in vacuo for 40 s. The resulting mixture was placed under an atmosphere of hydrogen and stirred at 25 ^0C for 7 h. The reaction mixture was filtered through Celite (CH_2Cl_2 wash). The solvent was removed in vacuo. Chromatography on silica gel eluted with EtOAc/Hex (2/8) afforded **2** (77 mg, 98%). ^{1}NMR DMSO-d_6) δ 10.36 (s, 1H), 8.04 (d, 1H, J=8.2 Hz), 7.72 (d, 1H, J=8.2 Hz), 7.61 (br s, 1H), 7.45 (t, 1H, J=8.4 Hz), 7.261 (t, 1H, J=8.4 Hz), 4.06 (m, 4H), 3.73 (m, 1H), 1.52 (s, 9H).

Synthesis of Compound (4). A solution of **2** (35 mg, 0.1 mmol) in 4 M HCl-EtOAc (5 ml) was stirred at 25 ^{0}C under Ar for 30 min. The solvent was removed in vacuo. To the residue was added 5-acetylindone-2-carboxylic acid (24.4 mg, 0.12 mmol). A solution of EDC (22.9 mg, 0.12 mmol) in DMF (3 ml) was added and the reaction mixture was stirred at 25 ^{0}C for 5 h. The solvent was removed. The crude product was chromatographed on silica gel eluted with 10% MeOH in CH_2Cl_2 to give **4** (40.7mg, 93%). ^{1}HNMR DMSO-d_6) δ12.13 (s, 1H), 10.47 (s, 1H), 8.45 (s, 1H), 8.10 (d, 1H, J=8.4 Hz), 7.96 (br s, 1H), 7.85 (d, 2H, J=8.4 Hz), 7.54 (d, 1H, J=8.4 Hz), 7.51 (t, 1H, J=8.2 Hz), 7.36 (t, 1H, J=7.6), 7.35 (s, 1H), 4.81 (t, 1H, 11.2 Hz), 4.54 (dd, 1H, 8.8 Hz), 4.23 (m, 1H), 4.01 (dd, 1H, J=10.2 Hz), 3.86 (dd, 1H, J=10.7 Hz), 2.61 (s, 3H).

Synthesis of Compound (5). 4-Methyl-1-piperazinecarbonyl chloride hydrochloride (19.9 mg, 0.1 mmol) was added to a solution of **4** (20 mg, 0.05 mmol) and anhydrous pyridine (25 μml, 0.3 mmol) in 3% allyl alcohol in dry methylene chloride (4 ml) and the mixture was stirred for 16 h. Purification of the crude product on silica gel yielded **5** (23.6 mg, 91%). ^{1}NMR DMSO-d_6) δ 12.03 (s, 1H), 8.41 (s, 1H), 8.21 (s, 1H), 8.01 (d, 1H, J=8.4 Hz), 7.88 (d, 1H, J=8.4 Hz), 7.82 (dd, 1H, J=8.4 Hz), 7.58 (t, 1H, J=8.1 Hz), 7.51 (d, 1H, J=8.4 Hz), 7.46 (t, 1H, J=7.6 Hz), 7.37 (s, 1H), 4.86 (t, 1H, J=10.8 Hz), 4.57 (dd, 1H, J=10.8 Hz), 4.38 (m, 1H), 4.06 (dd, 1H, J=10.8 Hz), 3.86 (dd, 1H, J=11 Hz), 3.41 (br, 4H), 3.29 (br, 4H), 2.82 (s, 3H), 2.57 (s, 3H).

Synthesis of Compound (7). A solution of **5** (13 mg, 24 umol) and linker **6** (16.9 mg, 31 umol) in 5% acetic acid in dry methylene chloride (1 ml) was stirred for 30 min at 25 ^{0}C. The solvent was completely removed in vacuo and purified by HPLC (SymmetryPrep C_{18}, 7μm, 19 x 150 mm column) to give **7** (18.5 mg, 81%). MS: calcd for $C_{48}H_{57}ClN_8O_{11}$ (M+H) m/z 958.38, found 958.10.

EXAMPLE 7: Proliferation Assays

The biological activity of the cytotoxic compounds of the invention can be assayed using the well established ^{3}H-thymidine proliferation assay. This is a convenient method for quantitating cellular proliferation, as it evaluates DNA synthesis by measuring the

incorporation of exogenous radiolabeled ^{3}H-thymidine. This assay is highly reproducible and can accommodate large numbers of compounds.

To carry out the assay, promyelocytic leukemia cells, HL-60, are cultured in RPMI media containing 10 % heat inactivated fetal calf serum (FCS). On the day of the study, the cells are collected, washed and resuspended at a concentration of 0.5 x 10^6 cells/ml in RPMI containing 10% FCS. 100 µl of cell suspension is added to 96 well plates. Serial dilutions (3-fold increments) of doxorubicin (as a positive control) or test compounds are made and 100 µl of compounds are added per well. Finally 10 µl of a 100 µCi/ml ^{3}H-thymidine is added per well and the plates are incubated for 24 hours. The plates are harvested using a 96 well Harvester (Packard Instruments) and counted on a Packard Top Count counter. Four parameter logistic curves are fitted to the ^{3}H-thymidine incorporation as a function of drug molarity using Prism software to determine IC_{50} values.

The compounds of the invention generally have an IC_{50} value in the above assay of from about 1 pM to about 100 nM, preferably from about 10 pM to about 10 nM.

EXAMPLE 8: Conjugation of Drug-Linker Molecules to Antibodies

This example describes reaction conditions and methodologies for conjugating a drug-linker molecule of the invention (optionally including other groups, such as spacers, reactive functional groups and the like) to an antibody as a targeting agent, X^4. The conditions and methodologies are intended to be exemplary only and non-limiting. Other approaches for conjugating drug-linker molecules to antibodies are known in the art.

The conjugation method described herein is based on introduction of free thiol groups to the antibody through reaction of lysines of the antibody with 2-iminothiolane, followed by reaction of the drug-linker molecule with an active maleimide group. Initially the antibody to be conjugated was buffer exchanged into 0.1M phosphate buffer pH 8.0 containing 50mM NaCl, 2mM DTPA, pH 8.0 and concentrated to 5-10 mg/ml. Thiolation was achieved through addition of 2-iminothiolane to the antibody. The amount of 2-iminothiolane to be added was determined in preliminary experiments and varies from antibody to antibody. In the preliminary experiments, a titration of increasing amounts of 2-iminothiolane was added to the antibody, and following incubation with the antibody for one hour at room temperature, the antibody was desalted into 50mM HEPES buffer pH 6.0 using a Sephadex G-25 column and the number of

thiol groups introduced determined rapidly by reaction with dithiodipyridine (DTDP). Reaction of thiol groups with DTDP results in liberation of thiopyridine which is monitored at 324nm. Samples at a protein concentration of 0.5-1.0 mg/ml were used. The absorbance at 280nm was used to accurately determine the concentration of protein in the samples, and then an aliquot of each sample (0.9ml) was incubated with 0.1 ml DTDP (5mM stock solution in ethanol) for 10 minutes at room temperature. Blank samples of buffer alone plus DTDP were also incubated alongside. After 10 minutes, absorbance at 324nm was measured and the number of thiols present quantitated using an extinction coefficient for thiopyridine of $19800M^{-1}$.

Typically a thiolation level of three thiol groups per antibody is desired. For example, with one particular antibody this was achieved through adding a 15 fold molar excess of 2-iminothiolane followed by incubation at room temperature for 1 hour. Antibody to be conjugated was therefore incubated with 2-iminothiolane at the desired molar ratio and then desalted into conjugation buffer (50mM HEPES buffer pH 6.0 containing 5mM Glycine, 3% Glycerol and 2mM DTPA). The thiolated material was maintained on ice whilst the number of thiols introduced was quantitated as described above.

After verification of the number of thiols introduced, the drug-linker molecule containing an active maleimide group was added at a 3-fold molar excess per thiol. The conjugation reaction was carried out in conjugation buffer also containing a final concentration of 5% ethylene glycol dimethyl ether (or a suitable alternative solvent). Commonly, the drug-linker stock solution was dissolved in 90% ethylene glycol dimethyl ether, 10% dimethyl sulfoxide. For addition to antibody, the stock solution can be added directly to the thiolated antibody, which has enough ethylene glycol dimethyl ether added to bring the final concentration to 5%, or pre-diluted in conjugation buffer containing a final concentration of 10% ethylene glycol dimethyl ether, followed by addition to an equal volume of thiolated antibody.

The conjugation reaction was incubated at room temperature for 2 hours with mixing. Following incubation the reaction mix was centrifuged at 14000 RPM for 15 minutes and the pH was adjusted to 7.2 if purification was not immediate. Purification of conjugate was achieved through chromatography using a number of methods. Conjugate can be purified using size-exclusion chromatography on a Sephacryl S200 column pre-equilibrated with 50mM HEPES buffer pH 7.2 containing 5mM glycine, 50mM NaCl and 3% glycerol. Chromatography was carried out at a linear flow rate of 28 cm/h. Fractions containing conjugate were collected,

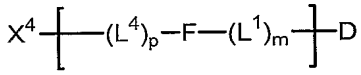
pooled and concentrated. Alternatively purification can be achieved through ion-exchange chromatography. Conditions vary from antibody to antibody and need to be optimized in each case. For example, antibody-drug conjugate reaction mix was applied to an SP-Sepharose column pre-equilibrated in 50mM HEPES, 5mM Glycine, 3%glycerol, pH 6.0. The antibody conjugate was eluted using a gradient of 0-1M NaCl in equilibration buffer. Fractions containing the conjugate were pooled, the pH was adjusted to 7.2 and the sample concentrated as required.

Each of the patent applications, patents, publications, and other published documents mentioned or referred to in this specification is herein incorporated by reference in its entirety, to the same extent as if each individual patent application, patent, publication, and other published document was specifically and individually indicated to be incorporated by reference.

While the present invention has been described with reference to the specific embodiments thereof, it should be understood by those skilled in the art that various changes may be made and equivalents may be substituted without departing from the true spirit and scope of the invention and the appended claims. In addition, many modifications may be made to adapt a particular situation, material, composition of matter, process, process step or steps, to the objective, spirit and scope of the present invention. All such modifications are intended to be within the scope of the claims appended hereto.

WHAT IS CLAIMED IS:

1. A compound of the formula



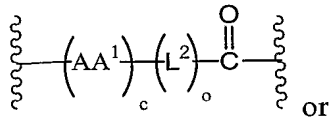
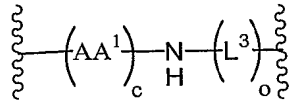
wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said functional group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

F is a linker comprising the structure:


or


wherein

AA^1 is one or more members independently selected from the group consisting of natural amino acids and unnatural α-amino acids;

c is an integer from 1 to 20;

L^2 is a self-immolative linker;

L^3 is a spacer group comprising a primary or secondary amine or a carboxyl functional group; wherein if L^3 is present, m is 0 and either the amine of L^3 forms an amide bond with a pendant carboxyl functional group of D or the carboxyl of L^3 forms an amide bond with a pendant amine functional group of D;

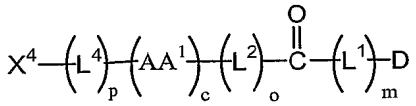
o is 0 or 1;

L^4 is a linker member, wherein L^4 does not comprise a carboxylic acyl group directly attached to the N-terminus of $(AA^1)_c$;

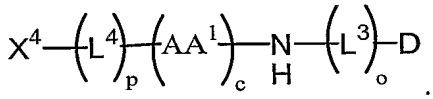
p is 0 or 1; and

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents.

2. The compound of claim 1, wherein the compound comprises the formula:



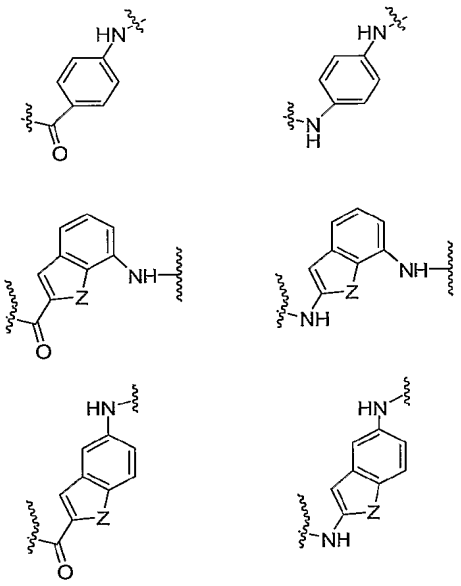
3. The compound of claim 1, wherein the compound comprises the formula:



4. The compound of claim 3, wherein L^3 comprises an aromatic group.

5. The compound of claim 4, wherein L^3 comprises a benzoic acid group, an aniline group, or an indole group.

6. The compound of claim 4, wherein $-L^3-NH-$ comprises a group having a structure selected from the group consisting of:



wherein Z is a member selected from O, S and NR^{23}, and

wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl.

7. The compound according to any one of claims 1 to 6, wherein L^4 comprises a non-cyclic moiety.

8. The compound according to any one of claims 1 to 7, wherein L^4 increases solubility of the compound as compared to the compound lacking L^4.

9. The compound according to any one of claims 1 to 8, wherein L^4 decreases aggregation of the compound as compared to the compound lacking L^4.

10. The compound according to any one of claims 1 to 9, wherein L^4 comprises a polyethylene glycol moiety.

11. The compound of claim 10, wherein the polyethylene glycol moiety contains 3-12 repeat units.

12. The compound of claim 11, wherein the polyethylene glycol moiety contains 2-6 repeat units.

13. The compound of claim 12, wherein the polyethylene glycol moiety contains 4 repeat units.

14. The compound according to any one of claims 1 to 13, wherein $(AA^1)_c$ is a peptide sequence cleavable by a protease expressed in tumor tissue.

15. The compound of claim 14, wherein the protease is a lysosomal protease.

16. The compound according to any one of claims 1 to 15, wherein c is an integer from 2 to 6.

17. The compound of claim 16, wherein c is 2, 3 or 4.

18. The compound of according to any one of claims claim 1 to 17, wherein the amino acid in $(AA^1)_c$ located closest to the drug moiety is selected from the group consisting of: Ala, Asn, Asp, Cit, Cys, Gln, Glu, Gly, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, and Val.

19. The compound of according to any one of claims claim 1 to 18, wherein $(AA^1)_c$ is a peptide sequence selected from the group consisting of Val-Cit, Val-Lys, Phe-Lys, Lys-Lys, Ala-Lys, Phe-Cit, Leu-Cit, Ile-Cit, Trp, Cit, Phe-Ala, Phe-N^9-tosyl-Arg, Phe-N^9-nitro-Arg, Phe-Phe-Lys, D-Phe-Phe-Lys, Gly-Phe-Lys, Leu-Ala-Leu, Ile-Ala-Leu, Val-Ala-Val, Ala-Leu-Ala-Leu (SEQ ID NO: 1), β-Ala-Leu-Ala-Leu (SEQ ID NO: 2) and Gly-Phe-Leu-Gly (SEQ ID NO: 3).

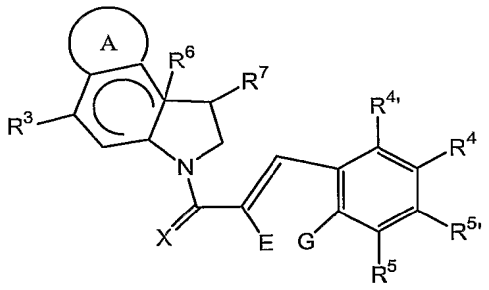
20. The compound according to any one of claims 1 to 19, wherein $(AA^1)_c$ is Val-Cit or Val-Lys.

21. The compound according to any one of claims 1 to 20, wherein D is a cytotoxic drug.

22. The compound of claim 21, wherein D comprises a chemically reactive functional group selected from the group consisting of a primary or secondary amine, hydroxyl, sulfhydryl and carboxyl.

23. The compound of claim 21, wherein D is selected from the group consisting of: duocarmycins, CC-1065, CBI-based duocarmycin analogues, MCBI-based duocarmycin analogues, CCBI-based duocarmycin analogues, doxorubicin, doxorubicin conjugates, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatins, dolestatin-10, combretastatin, calicheamicin, maytansine, maytansine analogues, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones, Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin, daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin, podophyllotoxin derivatives, etoposide, etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 -acetyl spermidine and camptothecin.

24. The compound according to any one of claims 1 to 23, wherein D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}{=}NR^{16}$, and $O(CH_2)_nN(CH_3)_2$

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

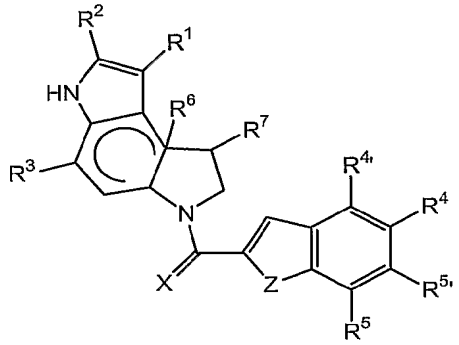
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2$- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F.

25. The compound of claim 24, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from group consisting of substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, and OR^9

in which

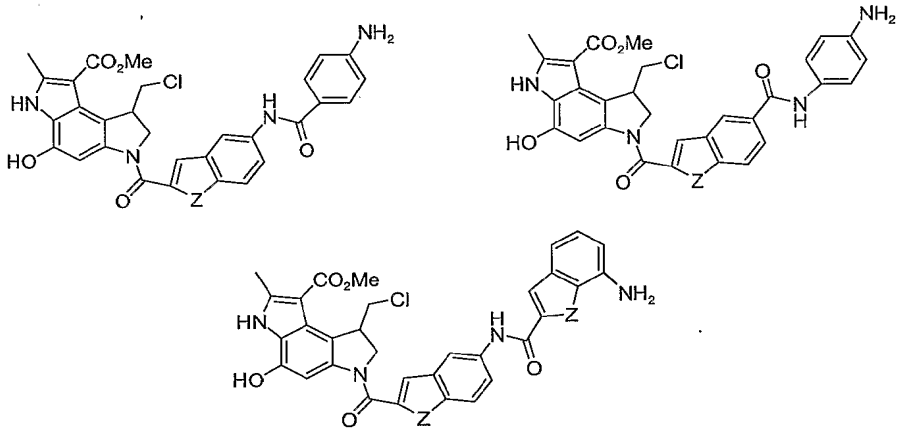
R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

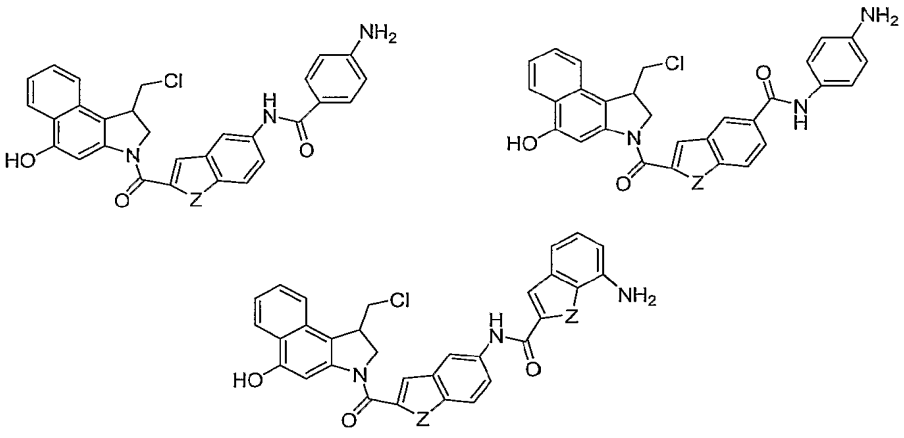
R^2 is H, substituted alkyl or unsubstituted lower alkyl;

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F.

26. The compound of claim 25, wherein R^2 is an unsubstituted lower alkyl.

27. The compound of claim 3, wherein NH_2-(L^3)-D has a structure selected from the group consisting of:





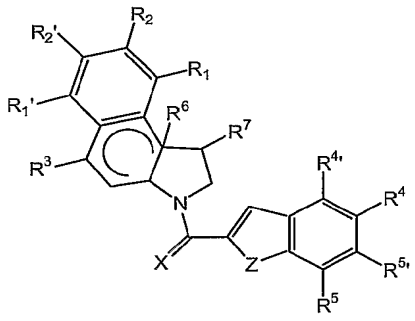
and

wherein Z is a member selected from O, S and NR^{23},

wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and

wherein the NH_2 group on each structure reacts with $(AA^1)_c$ to form $-(AA^1)_c$-NH-.

28. The compound of claim 24, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

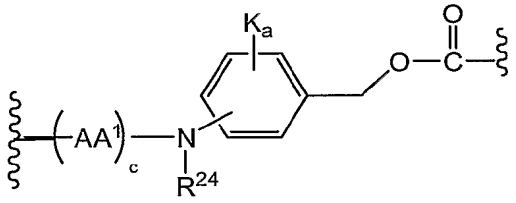
R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F.

29. The compound of claim 2, wherein F comprises the structure:



wherein

R^{24} is selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl;

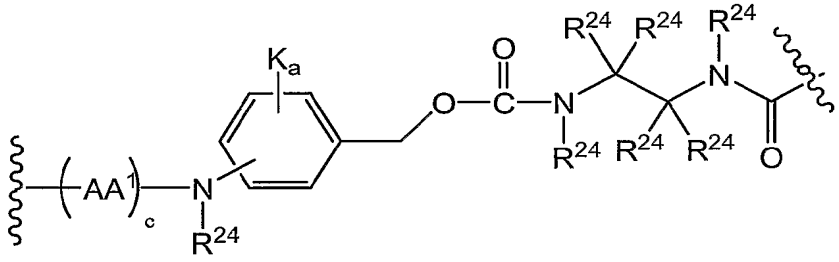
each K is a member independently selected from the group consisting of substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}

wherein

R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl; and

a is an integer of 0,1, 2, 3, or 4.

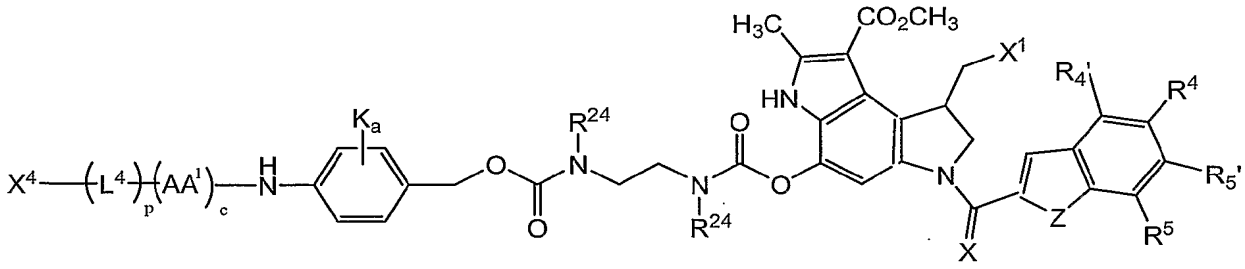
30. The compound of claim 29, wherein -F-$(L^1)_m$- comprises the structure:



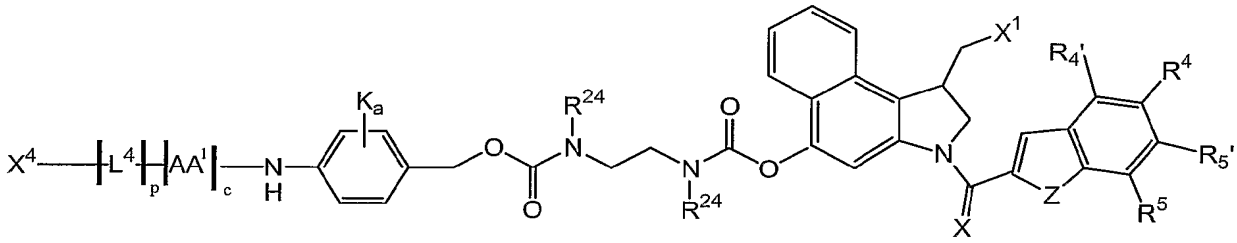
wherein

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl.

31. The compound of claim 29, having the structure:



or



wherein X^1 is a halogen;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and

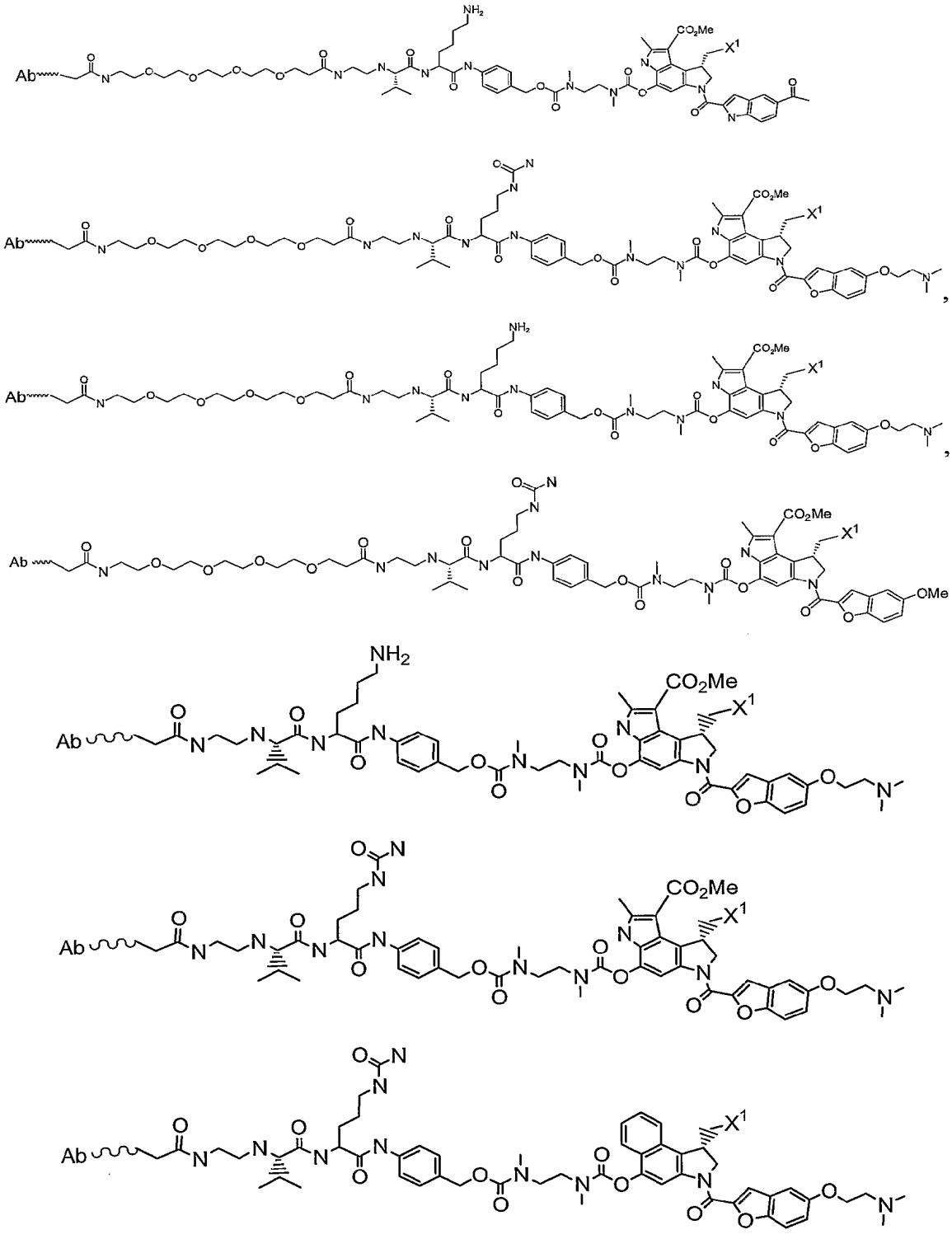
R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, OR^{15}, and $O(CH_2)_nN(CH_3)_2$

wherein

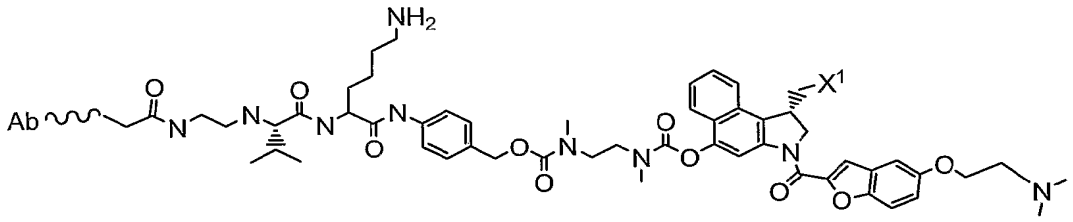
n is an integer from 1 to 20; and

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms.

32. The compound of claim 29, which is selected from the group consisting of:

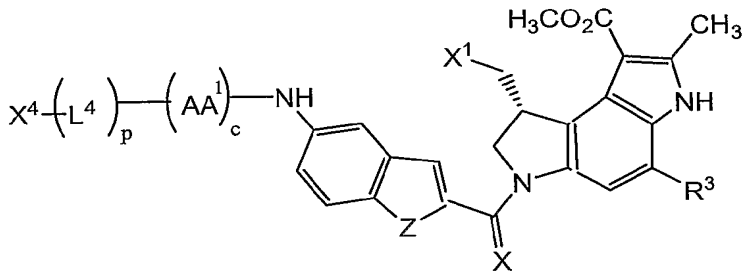


and

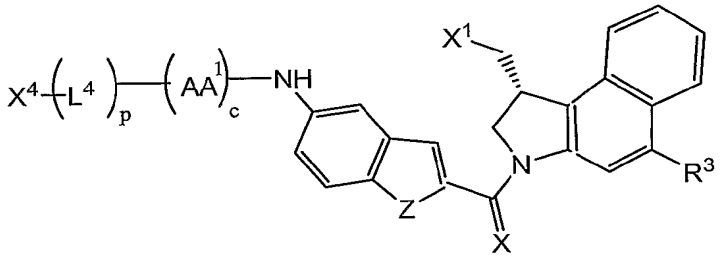


wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

33. The compound of claim 3, comprising the structure:



or



wherein X^1 is a leaving group;

Z and X are members independently selected from O, S and NR^{23},

wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl; and

R^3 is selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted

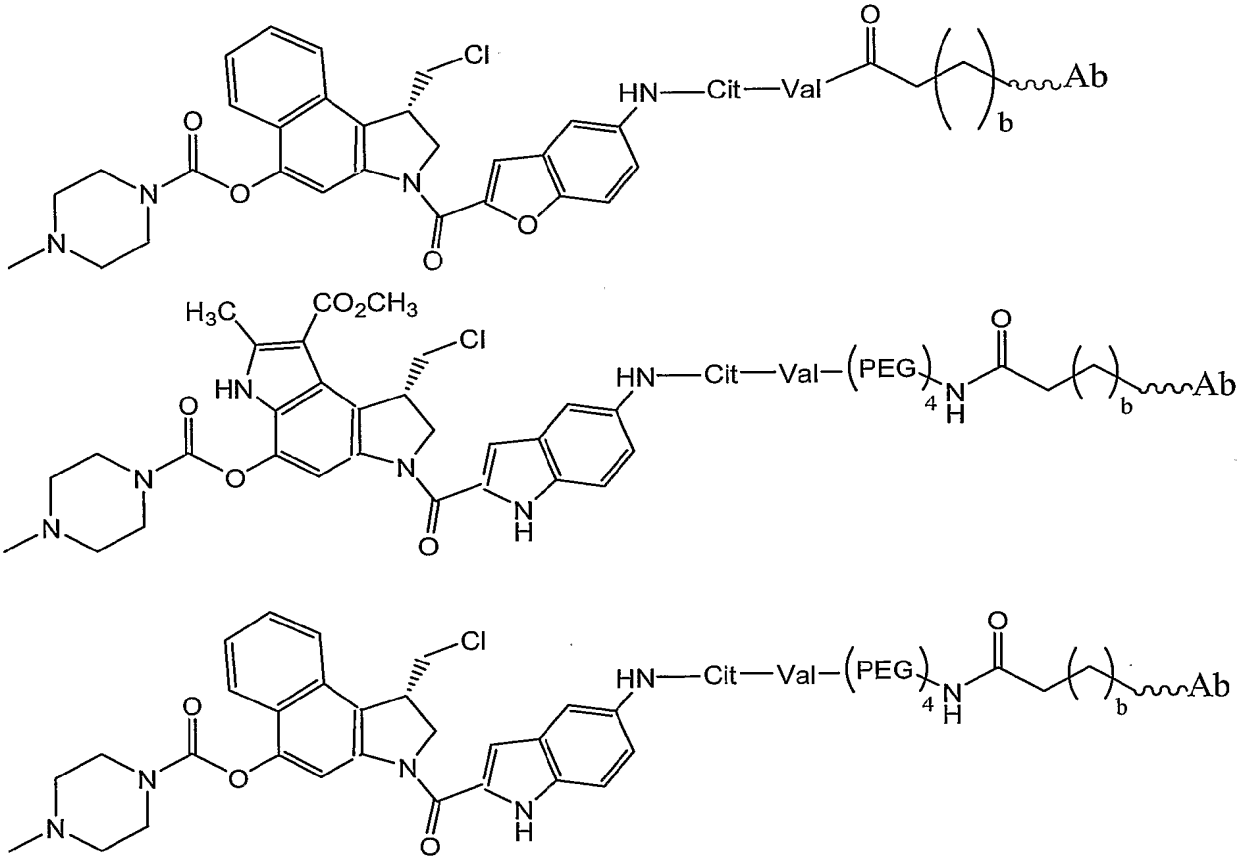
heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, OR^{15}, and $O(CH_2)_nN(CH_3)_2$

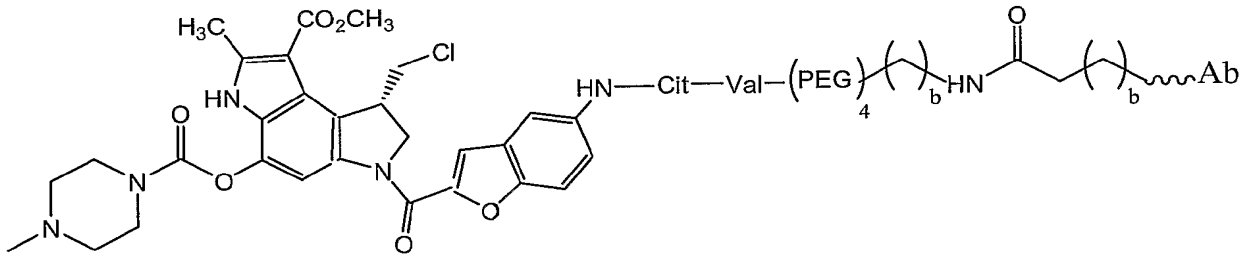
wherein

n is an integer from 1 to 20;

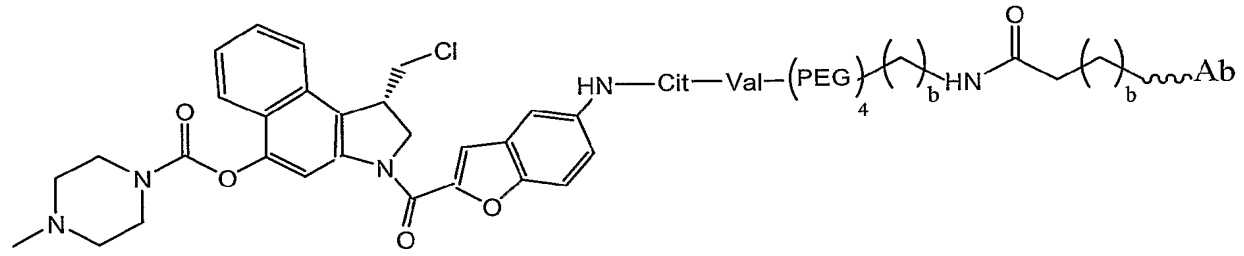
R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, and substituted or unsubstituted, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms.

34. The compound of claim 33, having the structure:





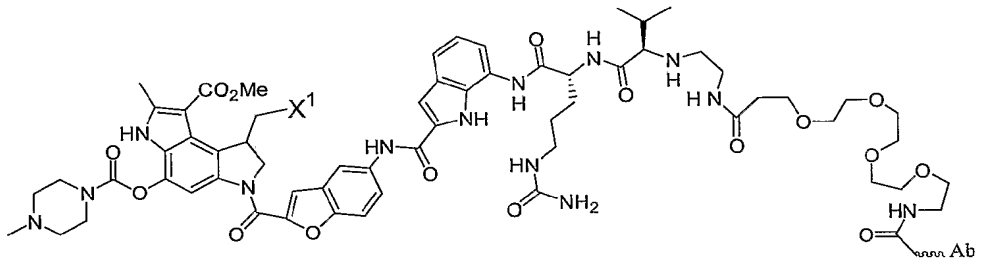
or

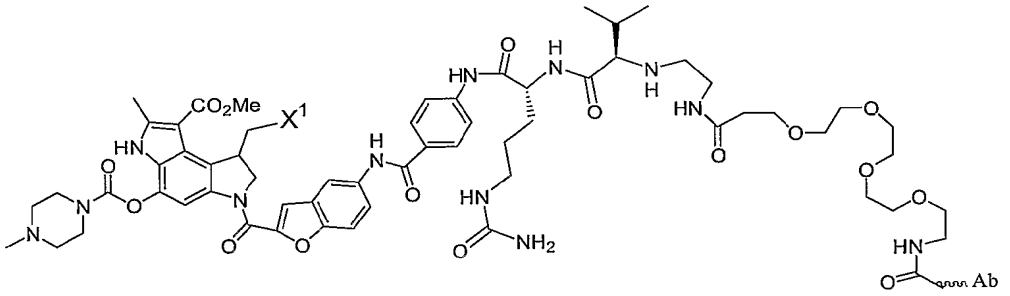


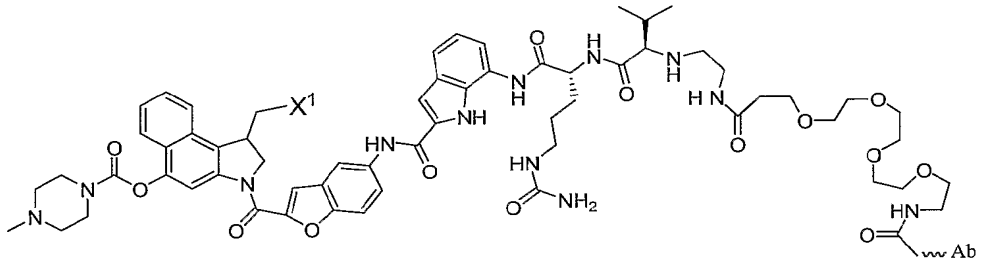
wherein each b is independently an integer from 0 to 20, and

wherein Ab is an antibody, or fragment thereof.

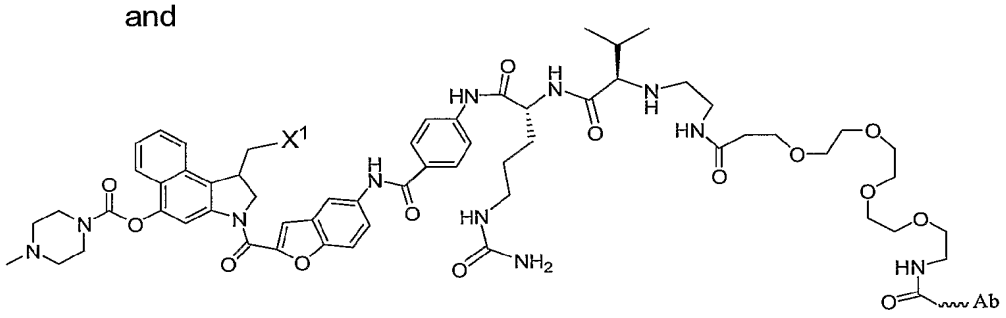
35. The compound of claim 3, which is selected from the group consisting of:





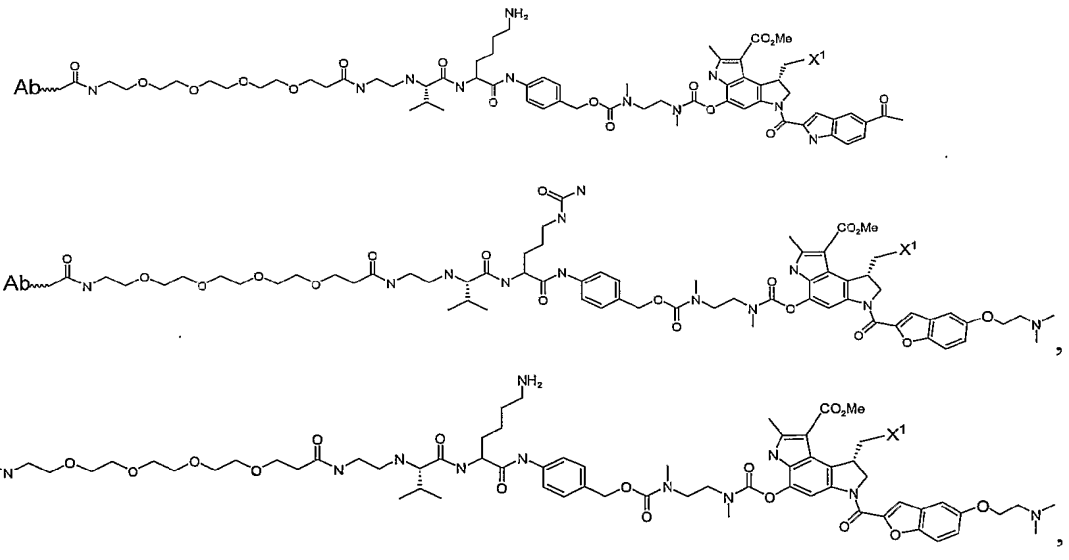


and

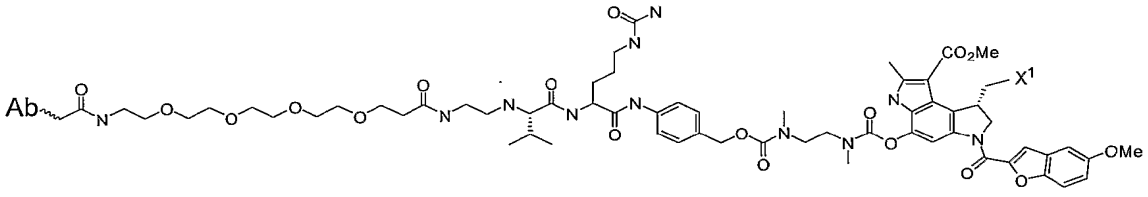


wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

36. The compound of claim 3, selected from the group consisting of:

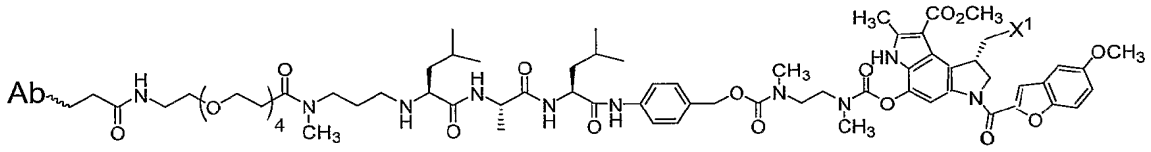


and



wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

37. The compound of claim 3, having the structure:



wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

38. A compound having the structure

$$X^4\text{——}(L^4)_p\text{—H—}(L^1)_m\text{——D}$$

wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said function group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

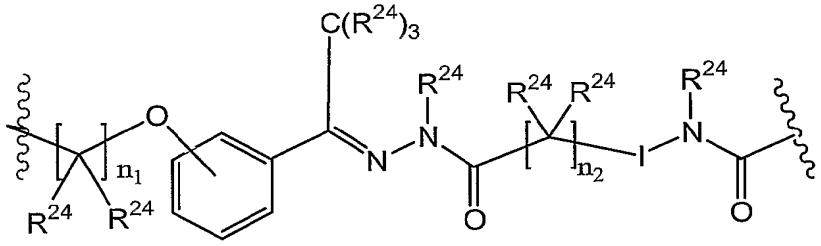
m is an integer selected from 0, 1, 2, 3, 4, 5, or 6;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents;

L^4 is a linker member;

p is 0 or 1;

H is a linker comprising the structure:



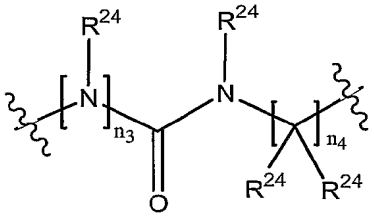
wherein

n_1 is an integer from 1 – 10;

n_2 is 0, 1, or 2;

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl; and

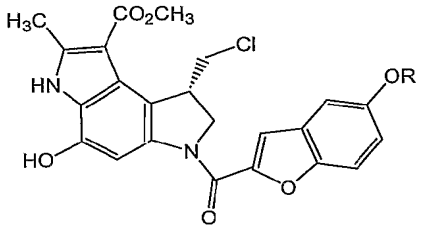
I is either a bond or:



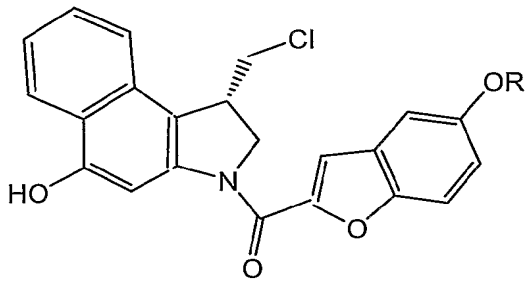
wherein n_3 is 0 or 1 with the proviso that when n_3 is 0, n_2 is not 0; and

n_4 is 1, 2, or 3,

wherein when I is a bond, n_1 is 3 and n_2 is 1, D can not be



or



where R is Me or CH_2- CH_2-NMe_2.

39. The compound of claim 38, wherein the substitution on the phenyl ring is a para substitution.

40. The compound according to any one of claims 38 and 39, wherein n_1 is 2, 3, or 4.

41. The compound of claim 40, wherein n_1 is 3.

42. The compound according to any one of claims 38 to 41, wherein n_2 is 1.

43. The compound of claim 42, wherein I is a bond.

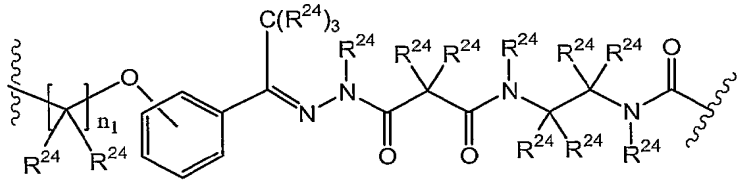
44. The compound according to any one of claims 38 to 43, wherein H forms a 6-membered self immolative linker upon cleavage

45. The compound of claim 42, wherein n_3 is 0 and n_4 is 2.

46. The compound according to any one of claims 38 to 43, wherein H forms two 5-membered self immolative linkers upon cleavage.

47. The compound according to any one of claims 38 to 43, wherein H forms a 5-membered self immolative linker, H forms a 7-membered self immolative linker, or H forms a 5-membered self immolative linker and a 6-membered self immolative linker, upon cleavage.

48. The compound according to any one of claims 38 to 43, wherein H comprises the structure



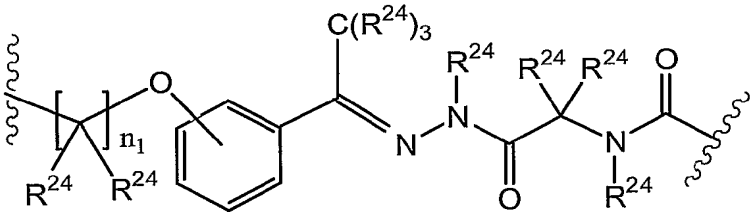
49. The compound of claim 48, wherein n_1 is 2, 3, or 4.

50. The compound of claim 48, wherein n_1 is 3.

51. The compound according to any one of claims 48 to 50, wherein each R_{24} is independently selected from CH_3 and H.

52. The compound according to any one of claims 48 to 51, wherein each R_{24} is H.

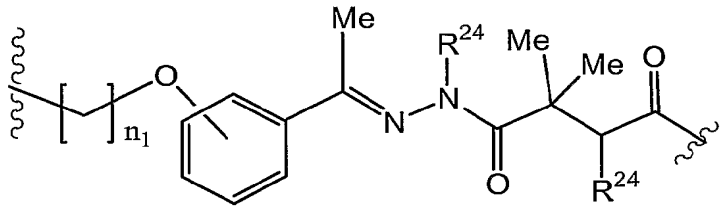
53. The compound according to any one of claims 38 to 43, wherein H has the structure:



54. The compound of claim 53, wherein n_1 is 3.

55. The compound of claim 53, wherein each R^{24} is independently selected from CH_3 and H.

56. The compound of claim 53, wherein H comprises the structure:



57. The compound of claim 53, wherein H comprises a geminal dimethyl substitution.

58. The compound of claim 56, wherein each R^{24} independently an H or a substituted or unsubstituted alkyl.

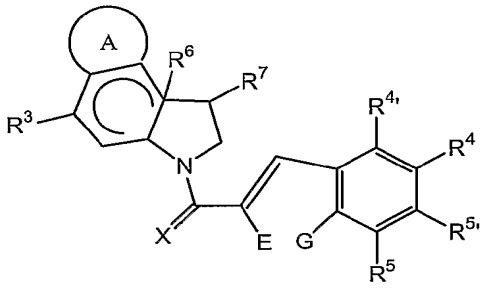
59. The compound according to any one of claims 38 to 58, wherein D is a cytotoxic drug.

60. The compound according to any one of claims 38 to 59, wherein D has a chemically reactive function group selected from the group consisting of a primary or secondary amine, hydroxyl, sulfhydryl and carboxyl.

61. The compound according to any one of claims 38 to 60, wherein D is selected from the group consisting of: duocarmycins, CC-1065, CBI-based duocarmycin analogues, MCBI-based duocarmycin analogues, CCBI-based duocarmycin analogues, doxorubicin, doxorubicin conjugates, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatins, dolestatin-10, combretastatin, calicheamicin, maytansine, maytansine analogues, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones, Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin, daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin,

tallysomycin, podophyllotoxin, podophyllotoxin derivatives, etoposide, etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 -acetyl spermidine and camptothecin.

62. The compound according to any one of claims 38 to 61, wherein D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

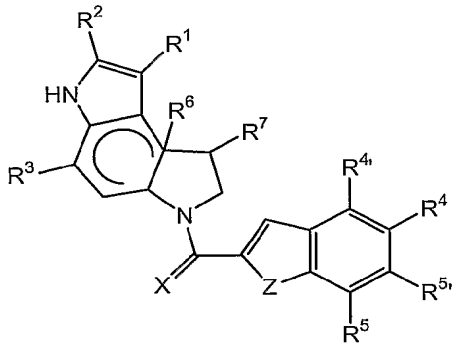
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2-$ joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to H.

63. The compound of claim 62, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from group consisting of substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, and OR^9

in which

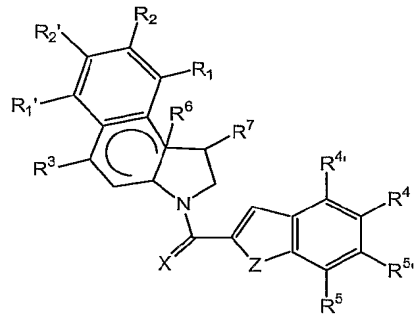
R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^2 is H, substituted alkyl or unsubstituted lower alkyl;

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to H.

64. The compound of claim 63, wherein R^2 is an unsubstituted lower alkyl.

65. The compound of claim 62, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to H.

66. The compound according to any one of claims 38 to 65, wherein L^4 comprises a non-cyclic moiety.

67. The compound according to any one of claims 38 to 66, wherein L^4 increases solubility of the compound as compared to the compound lacking L^4.

68. The compound according to any one of claims 38 to 67, wherein L^4 decreases aggregation of the compound as compared to the compound lacking L^4.

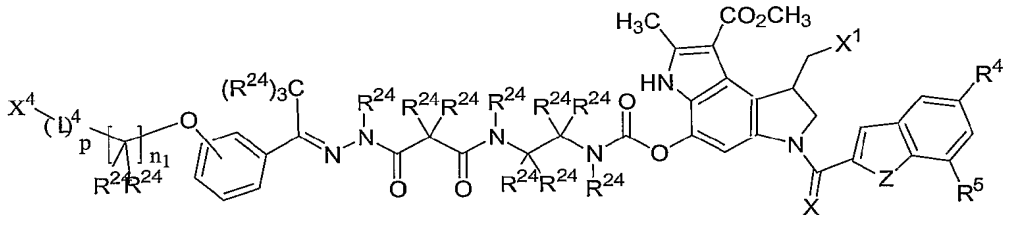
69. The compound according to any one of claims 38 to 68, wherein L^4 comprises a polyethylene glycol moiety.

70. The compound of claim 69, wherein the polyethylene glycol moiety contains 3-12 repeat units.

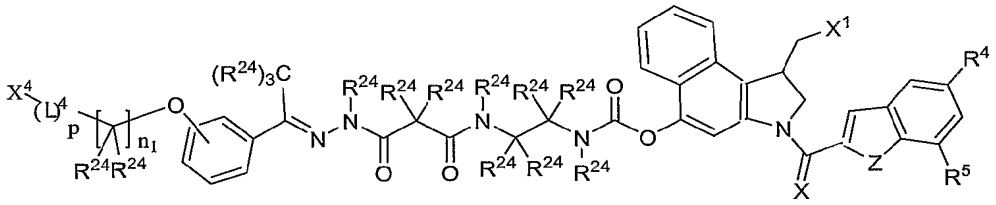
71. The compound of claim 70, wherein the polyethylene glycol moiety contains 2-6 repeat units.

72. The compound of claim 71, wherein the polyethylene glycol moiety contains 4 repeat units.

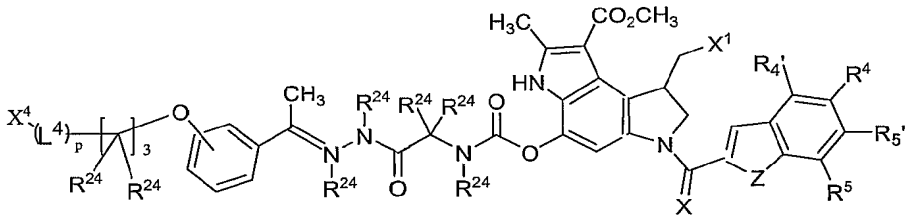
73. The compound of claim 63, having the structure:



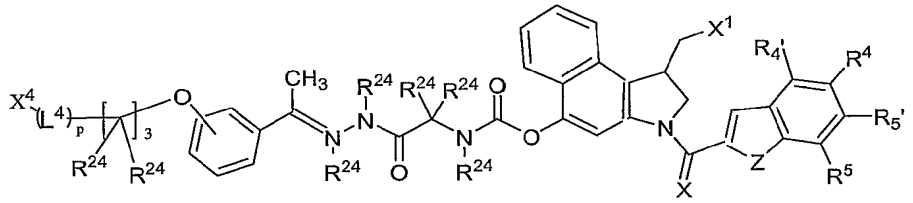
or



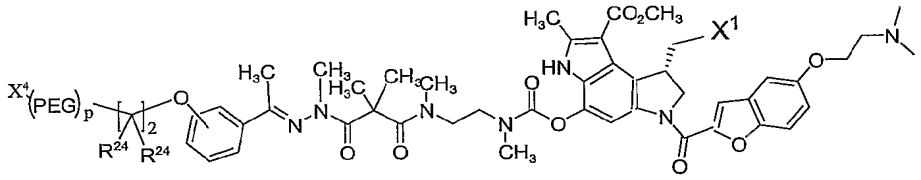
74. The compound of claim 63, having the structure:



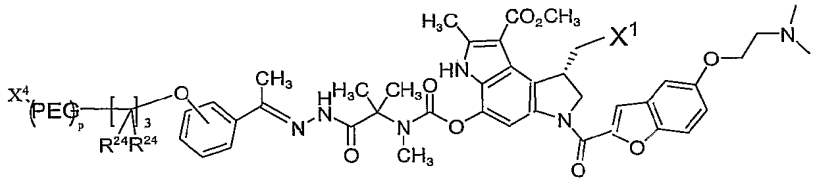
or



75. The compound of claim 63, having the structure:

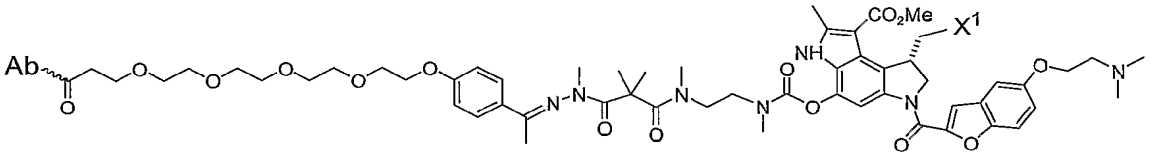


or



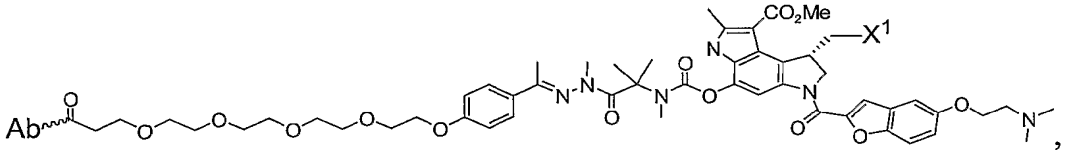
wherein PEG is a polyethylene glycol moiety and X^1 is Cl or Br.

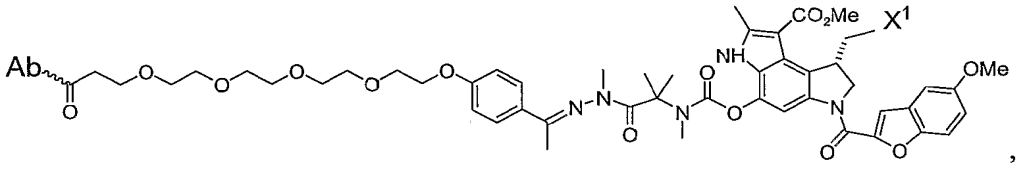
76. The compound of claim 63, having the structure:



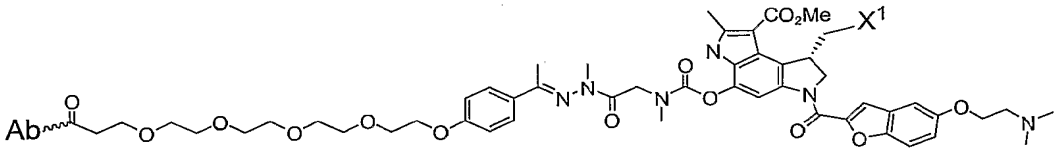
wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

77. The compound of claim 63, having a structure selected from the group consisting of:



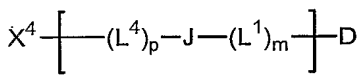


and



wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

78. A compound of the formula



wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said function group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

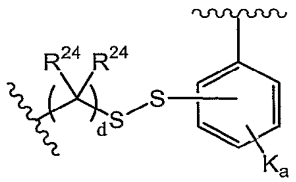
m is an integer selected from 0, 1, 2, 3, 4, 5, or 6;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents;

L^4 is a linker member;

P is 0 or 1;

J is a linker comprising the structure:



wherein

each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl;

each K is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{21}R^{22}$, $NR^{21}COR^{22}$, $OCONR^{21}R^{22}$, $OCOR^{21}$, and OR^{21}

wherein

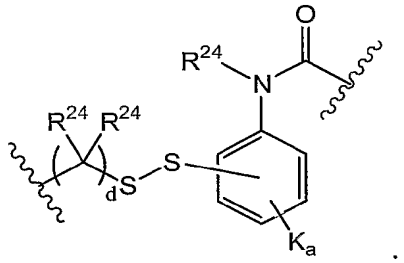
R^{21} and R^{22} are independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl,

substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl and unsubstituted heterocycloalkyl;

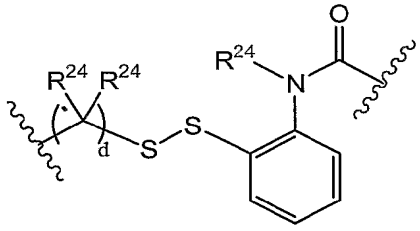
a is an integer of 0,1, 2, 3, or 4; and

d is an integer of 0, 1, 2, 3, 4, 5, or 6.

79. The compound of claim 78, wherein J comprises the structure:

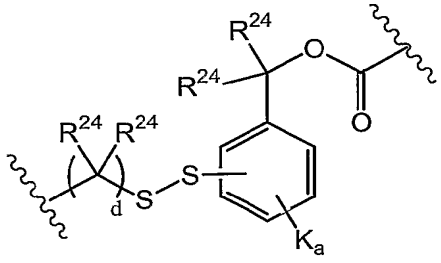


80. The compound of claim 78, wherein J comprises the structure:

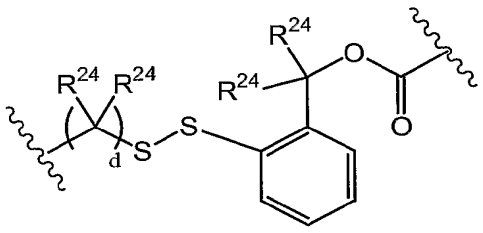


81. The compound of claim 80, wherein d is 1 or 2.

82. The compound of claim 78, wherein J comprises the structure:



83. The compound of claim 82, wherein J comprises the structure:

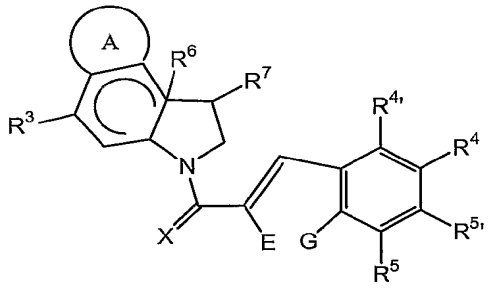


84. The compound according to any one of claims 78 to 83, wherein D is a cytotoxic drug.

85. The compound according to any one of claims 78 to 84, wherein D has a chemically reactive function group selected from the group consisting of a primary or secondary amine, hydroxyl, sulfhydryl and carboxyl.

86. The compound according to any one of claims 78 to 85, wherein D is selected from the group consisting of: duocarmycins, CC-1065, CBI-based duocarmycin analogues, MCBI-based duocarmycin analogues, CCBI-based duocarmycin analogues, doxorubicin, doxorubicin conjugates, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatins, dolestatin-10, combretastatin, calicheamicin, maytansine, maytansine analogues, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones, Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin, daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin, podophyllotoxin derivatives, etoposide, etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 -acetyl spermidine and camptothecin.

87. The compound according to any one of claims 78 to 86, wherein D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached

are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

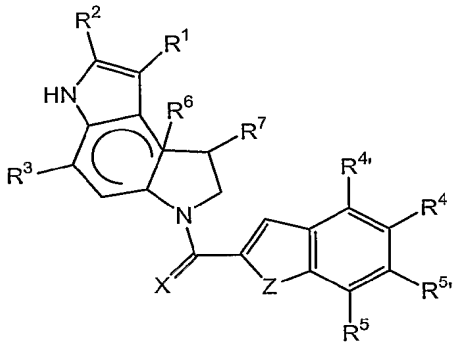
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2-$ joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to J.

88. The compound of claim 87, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from group consisting of substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, and OR^9

in which

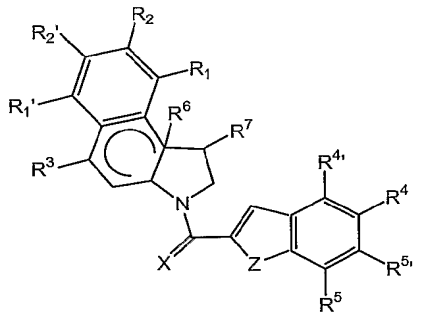
R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^2 is H, substituted alkyl or unsubstituted lower alkyl;

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to J.

89. The compound of claim 88, wherein R^2 is an unsubstituted lower alkyl.

90. The compound of claim 87, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to J.

91. The compound according to any one of claims 78 to 90, wherein L^4 comprises a non-cyclic moiety.

92. The compound according to any one of claims 78 to 91, wherein L^4 increases solubility of the compound as compared to the compound lacking L^4.

93. The compound according to any one of claims 78 to 92, wherein L^4 decreases aggregation of the compound as compared to the compound lacking L^4.

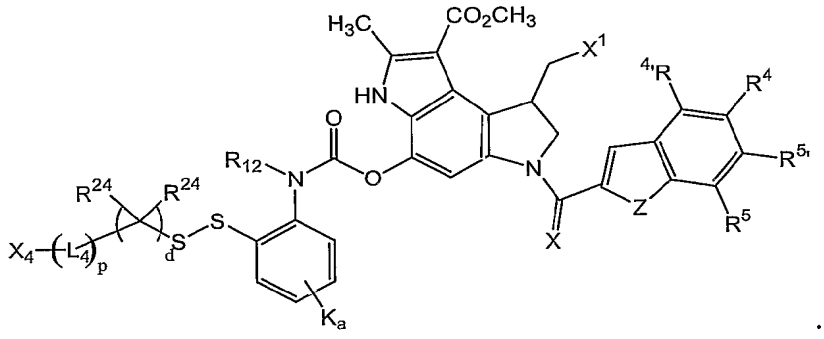
94. The compound according to any one of claims 78 to 93, wherein L^4 comprises a polyethylene glycol moiety.

95. The compound of claim 94, wherein the polyethylene glycol moiety contains 3-12 repeat units.

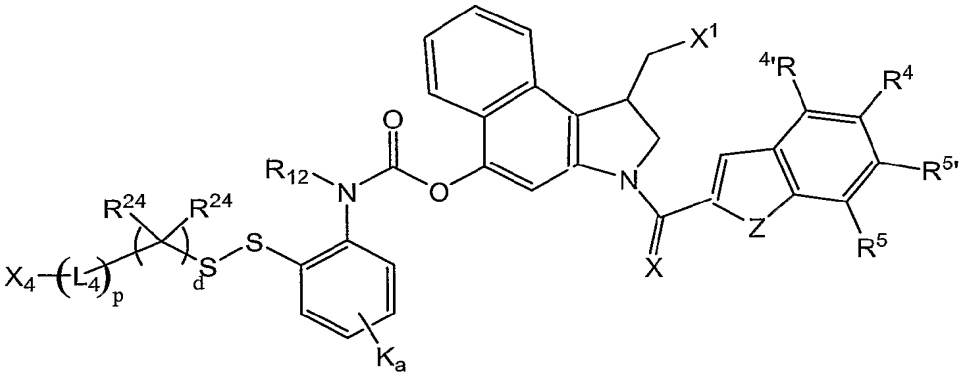
96. The compound of claim 95, wherein the polyethylene glycol moiety contains 2-6 repeat units.

97. The compound of claim 96, wherein the polyethylene glycol moiety contains 4 repeat units.

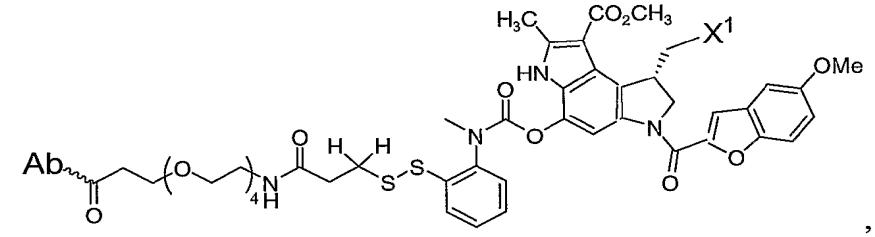
98. The compound of claim 88, having the structure:



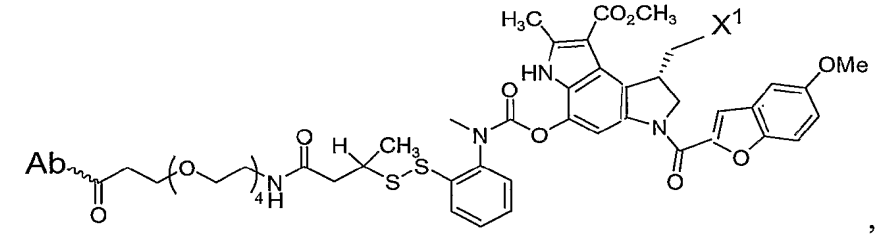
or



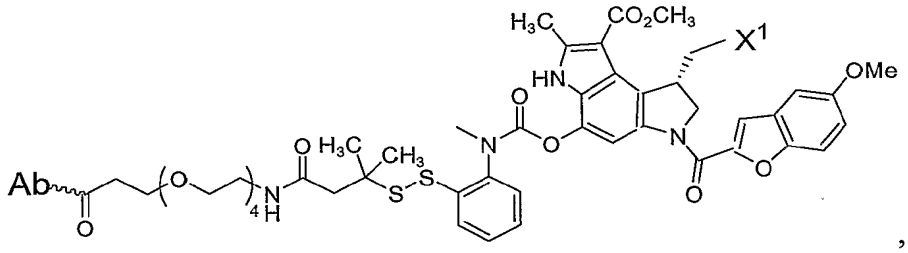
99. A compound of claim 88, having a structure selected from the group consisting of:



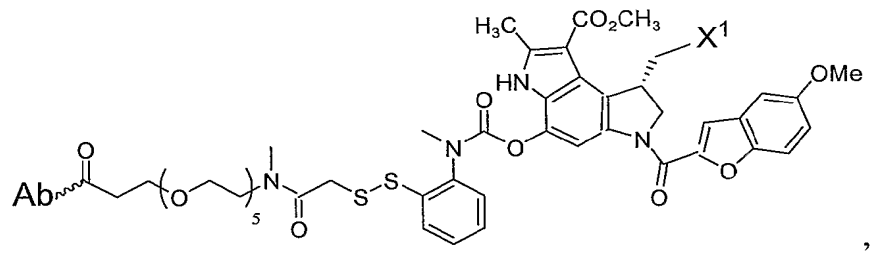
,

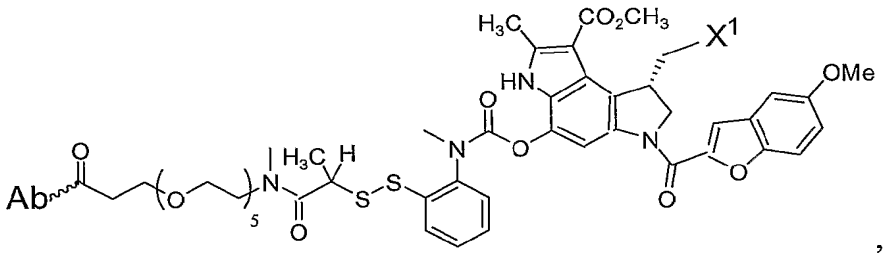


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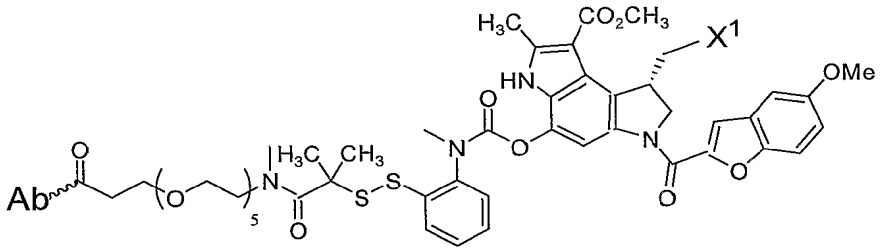


,





and



wherein X^1 is Cl or Br, and Ab is an antibody, or fragment thereof.

100. A compound having the structure

$$X^4\text{——}(L^4)_p\text{—H—}(L^1)_m\text{——D}$$

wherein

D is a drug moiety having pendant to the backbone thereof a chemically reactive functional group, said function group selected from the group consisting of a primary or secondary amine, hydroxyl, thiol, carboxyl, aldehyde, and a ketone;

L^1 is a self-immolative linker;

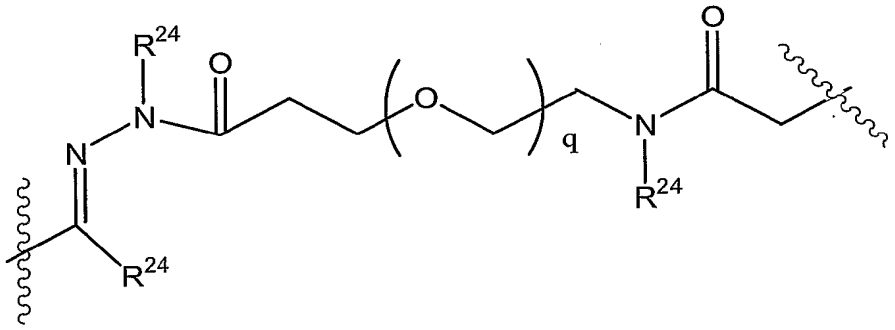
m is an integer selected from 0, 1, 2, 3, 4, 5, or 6;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents;

L^4 is a linker member;

p is 0 or 1;

H is a linker comprising the structure:



where q is 0, 1,2, 3, 4, 5, or 6; and

wherein each R^{24} is a member independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, and unsubstituted heteroalkyl. This hydrazine structure can also form five-, six-, or seven-membered rings and additional components can be added to form multiple rings.

101. The compound of claim 100, wherein H forms a 6-membered self immolative linker upon cleavage

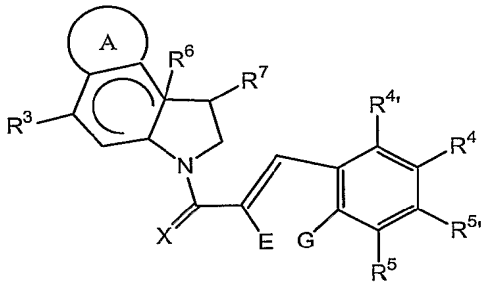
102. The compound of claim 100, wherein H forms two 5-membered self immolative linkers upon cleavage.

103. The compound according to any one of claims 100 to 102, wherein D is a cytotoxic drug.

104. The compound according to any one of claims 100 to 103, wherein D has a chemically reactive function group selected from the group consisting of a primary or secondary amine, hydroxyl, sulfhydryl and carboxyl.

105. The compound according to any one of claims 100 to 104, wherein D is selected from the group consisting of: duocarmycins, CC-1065, CBI-based duocarmycin analogues, MCBI-based duocarmycin analogues, CCBI-based duocarmycin analogues, doxorubicin, doxorubicin conjugates, morpholino-doxorubicin, cyanomorpholino-doxorubicin, dolastatins, dolestatin-10, combretastatin, calicheamicin, maytansine, maytansine analogues, DM-1, auristatin E, auristatin EB (AEB), auristatin EFP (AEFP), monomethyl auristatin E (MMAE), 5-benzoylvaleric acid-AE ester (AEVB), tubulysins, disorazole, epothilones, Paclitaxel, docetaxel, SN-38, Topotecan, rhizoxin, echinomycin, colchicine, vinblastin, vindesine, estramustine, cemadotin, eleutherobin, methotrexate, methopterin, dichloromethotrexate, 5-fluorouracil, 6-mercaptopurine, cytosine arabinoside, melphalan, leurosine, leurosideine, actinomycin, daunorubicin, daunorubicin conjugates, mitomycin C, mitomycin A, carminomycin, aminopterin, tallysomycin, podophyllotoxin, podophyllotoxin derivatives, etoposide, etoposide phosphate, vincristine, taxol, taxotere retinoic acid, butyric acid, N^8 -acetyl spermidine and camptothecin.

106. The compound according to any one of claims 100 to 105, wherein D comprises a structure:



wherein the ring system A is a member selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl groups;

E and G are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, a heteroatom, a single bond, or E and G are joined to form a ring system selected from substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl and substituted or unsubstituted heterocycloalkyl;

X is a member selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^3 is a member selected from the group consisting of (=O), SR^{11}, NHR^{11} and OR^{11},

wherein

R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$,

in which

R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nN(CH_3)_2$

wherein

n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

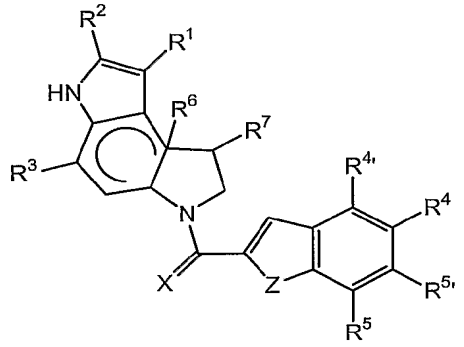
R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2$- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to F.

107. The compound of claim 106, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from group consisting of substituted alkyl, unsubstituted alkyl, NR^9R^{10}, NR^9NHR^{10}, and OR^9

in which

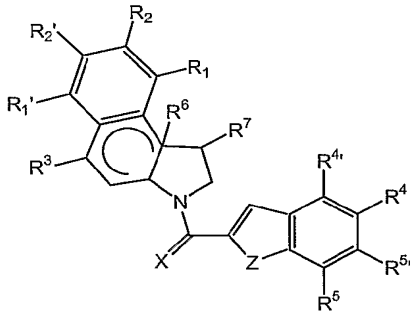
R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl; and

R^2 is H, substituted alkyl or unsubstituted lower alkyl;

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to H.

108. The compound of claim 107, wherein R^2 is an unsubstituted lower alkyl.

109. The compound of claim 106, wherein D has the structure:



wherein

Z is a member selected from O, S and NR^{23}

wherein

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$, wherein R^8 is a member selected from NR^9R^{10} and OR^9,

in which

R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy; and

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

wherein at least one of R^{11}, R^{12}, R^{13}, R^{15} or R^{16} links said drug to L^1, if present, or to H.

110. The compound according to any one of claims 100 to 109, wherein L^4 comprises a non-cyclic moiety.

111. The compound according to any one of claims 100 to 110, wherein L^4 increases solubility of the compound as compared to the compound lacking L^4.

112. The compound according to any one of claims 100 to 111, wherein L^4 decreases aggregation of the compound as compared to the compound lacking L^4.

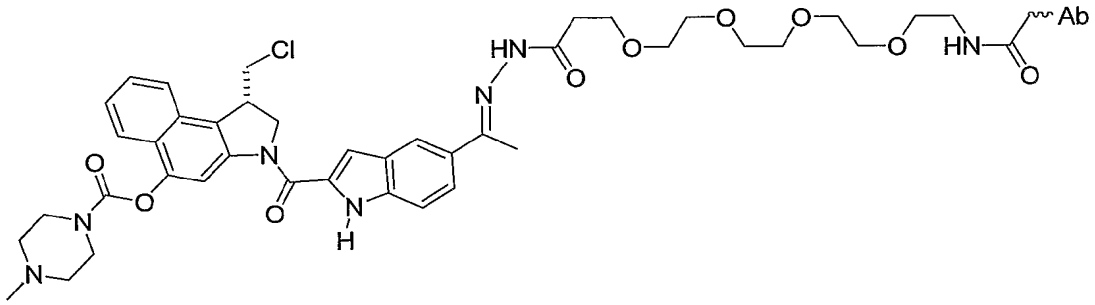
113. The compound according to any one of claims 100 to 112, wherein L^4 comprises a polyethylene glycol moiety.

114. The compound of claim 113, wherein the polyethylene glycol moiety contains 3-12 repeat units.

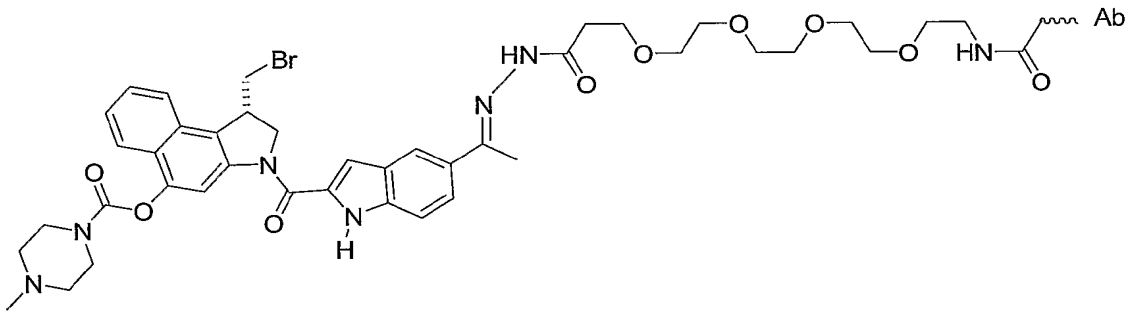
115. The compound of claim 114, wherein the polyethylene glycol moiety contains 2-6 repeat units.

116. The compound of claim 115, wherein the polyethylene glycol moiety contains 4 repeat units.

117. The compound of claim 109, having the structure:

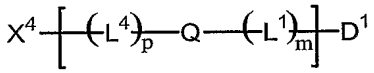


or



wherein Ab is an antibody, or fragment thereof.

118. A compound of the formula:



wherein

L^1 is a self-immolative linker;

m is an integer 0, 1, 2, 3, 4, 5, or 6;

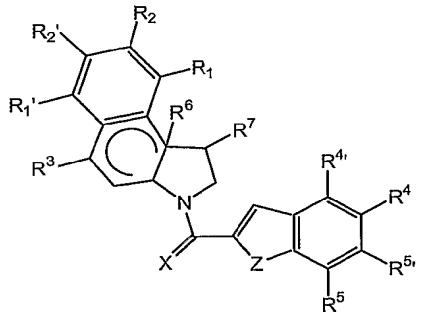
L^4 is a linker member, wherein L^4 does not comprise a carboxylic acyl group directly attached to the N-terminus of $(AA^1)_c$;

p is 0 or 1;

X^4 is a member selected from the group consisting of protected reactive functional groups, unprotected reactive functional groups, detectable labels, and targeting agents;

Q is a cleavable linker; and

D^1 is a a drug having the following formula:



wherein X and Z are members independently selected from O, S and NR^{23};

R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8,

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$,

wherein R^8 is a member selected from NR^9R^{10} and OR^9 and R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl or cyano or alkoxy;

$R^{2'}$ is H, or substituted or unsubstituted lower alkyl or unsubstituted heteroalkyl,

R^3 is a member selected from the group consisting of SR^{11}, NHR^{11} and OR^{11}, wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$, in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, wherein R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

wherein at least one of R^{11}, R^{12}, and R^{13} links said drug to L^1, if present, or to Q,

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or $-CH_2$- joined in said cyclopropyl ring with R^6, wherein

X^1 is a leaving group,

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted

heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nNR^{24}R^{25}$ wherein n is an integer from 1 to 20;

wherein R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

and R^{24} and R^{25} are independently selected from unsubstituted alkyl, and

wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ is $O(CH_2)_nNR^{24}R^{25}$.

119. The compound of claim 118, wherein n is 2.

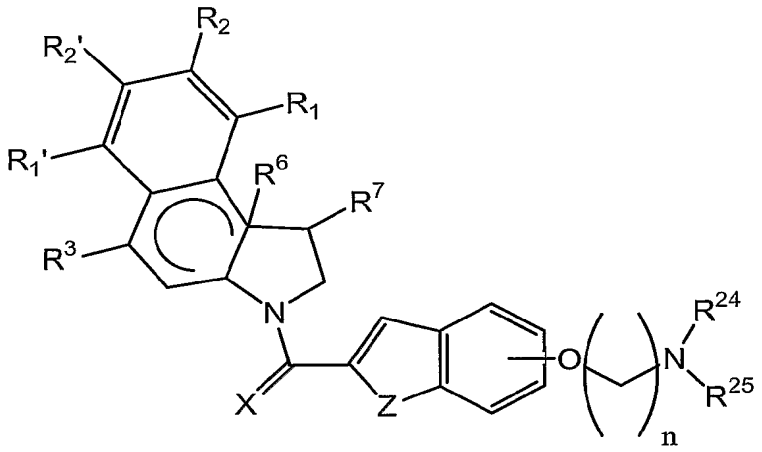
120. The compound according to any one of claims 118 and 119, wherein R^{24} and R^{25} are methyl.

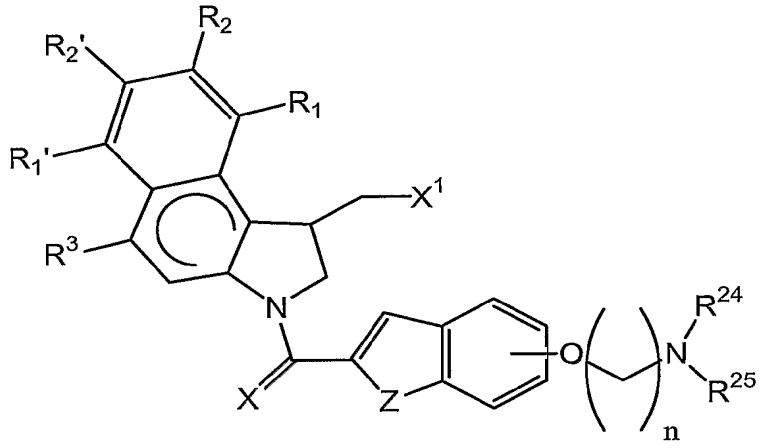
121. The compound according to any one of claims 118 to 120, wherein $R^{4'}$, R^5, and $R^{5'}$ are H and R^4 is $O(CH_2)_nNR^{24}R^{25}$.

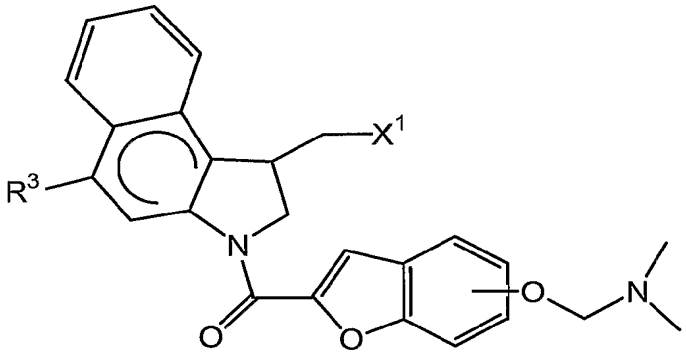
122. The compound of claim 121, wherein R^4 is $O(CH_2)_2N(CH_3)_2$.

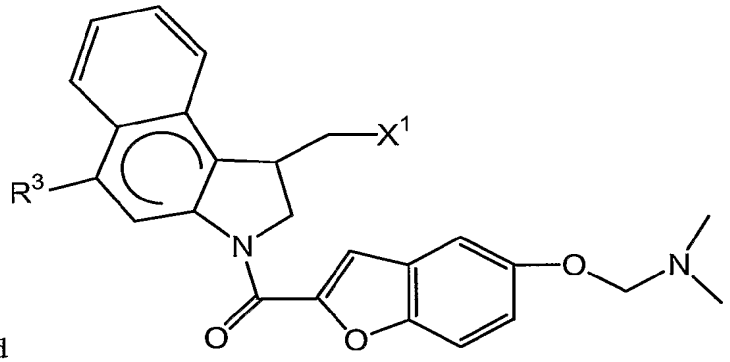
123. The compound according to any one of claims 118 to 122, wherein R^1, $R^{1'}$, R^2, and $R^{2'}$ are H.

124. The compound according to any one of claims 118 to 123, wherein the drug D^1 has a formula selected from:

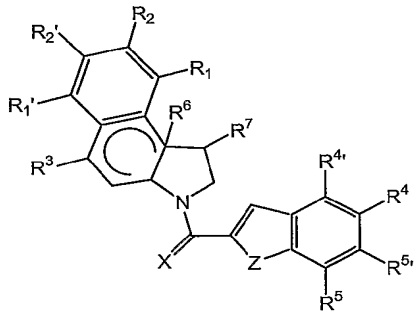






and 

125. A compound of the formula



wherein X and Z are independently selected from O, S and NR^{23} wherein R^{23} is a member selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, and acyl;

R^1 is H, substituted or unsubstituted lower alkyl, $C(O)R^8$, or CO_2R^8,

$R^{1'}$ is H, substituted or unsubstituted lower alkyl, or $C(O)R^8$,

each R^8 is a member independently selected from NR^9R^{10} and OR^9 and R^9 and R^{10} are members independently selected from H, substituted or unsubstituted alkyl and substituted or unsubstituted heteroalkyl;

R^2 is H, substituted or unsubstituted lower alkyl, unsubstituted heteroalkyl, cyano, or alkoxy;

$R^{2'}$ is H, substituted or unsubstituted lower alkyl, or unsubstituted heteroalkyl,

R^3 is a member selected from the group consisting of SR^{11}, NHR^{11} and OR^{11}, wherein R^{11} is a member selected from the group consisting of H, substituted alkyl, unsubstituted alkyl,

substituted heteroalkyl, unsubstituted heteroalkyl, diphosphates, triphosphates, acyl, $C(O)R^{12}R^{13}$, $C(O)OR^{12}$, $C(O)NR^{12}R^{13}$, $P(O)(OR^{12})_2$, , $C(O)CHR^{12}R^{13}$, SR^{12} and $SiR^{12}R^{13}R^{14}$, in which R^{12}, R^{13}, and R^{14} are members independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl and substituted or unsubstituted aryl, or R^{12} and R^{13} together with the nitrogen or carbon atom to which they are attached are joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members;

R^6 is a single bond which is either present or absent and when present R^6 and R^7 are joined to form a cyclopropyl ring; and

R^7 is CH_2-X^1 or –CH_2- joined in said cyclopropyl ring with R^6, wherein X^1 is a leaving group,

R^4 , $R^{4'}$, R^5 and $R^{5'}$ are members independently selected from the group consisting of H, substituted alkyl, unsubstituted alkyl, substituted aryl, unsubstituted aryl, substituted heteroaryl, unsubstituted heteroaryl, substituted heterocycloalkyl, unsubstituted heterocycloalkyl, halogen, NO_2, $NR^{15}R^{16}$, $NC(O)R^{15}$, $OC(O)NR^{15}R^{16}$, $OC(O)OR^{15}$, $C(O)R^{15}$, SR^{15}, OR^{15}, $CR^{15}=NR^{16}$, and $O(CH_2)_nNR^{24}R^{25}$ wherein n is an integer from 1 to 20;

R^{15} and R^{16} are independently selected from H, substituted or unsubstituted alkyl, substituted or unsubstituted heteroalkyl, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocycloalkyl, and substituted or unsubstituted peptidyl, wherein R^{15} and R^{16} together with the nitrogen atom to which they are attached are optionally joined to form a substituted or unsubstituted heterocycloalkyl ring system having from 4 to 6 members, optionally containing two or more heteroatoms;

and R^{24} and R^{25} are independently selected from unsubstituted alkyl, and

wherein at least one of R^4 , $R^{4'}$, R^5 and $R^{5'}$ is $O(CH_2)_nNR^{24}R^{25}$.

126. The compound of claim 125, wherein R^4 is $O(CH_2)_nNR^{24}R^{25}$.

127. The compound of claim 126, wherein R^4 is $O(CH_2)_nN(CH_3)_2$.

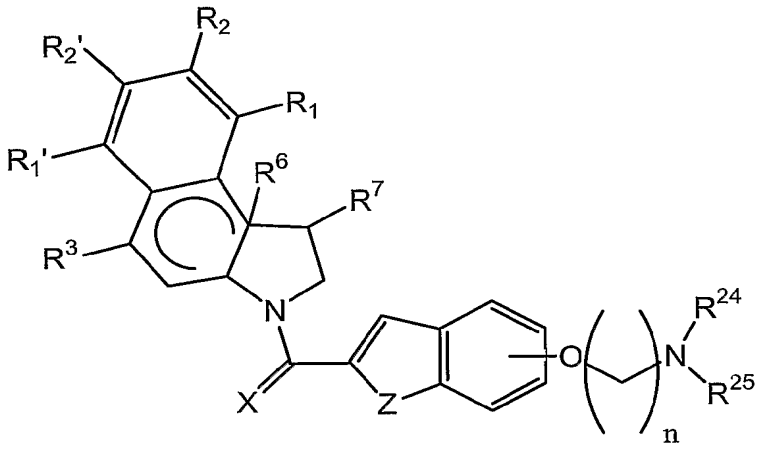
128. The compound of claim127, wherein $R^{4'}$, R^5 and $R^{5'}$ are H.

129. The compound according to any one of claims 125 to 128, wherein R^6 is absent and R^7 is CH_2-X^1, wherein X^1 is F, Cl, or Br.

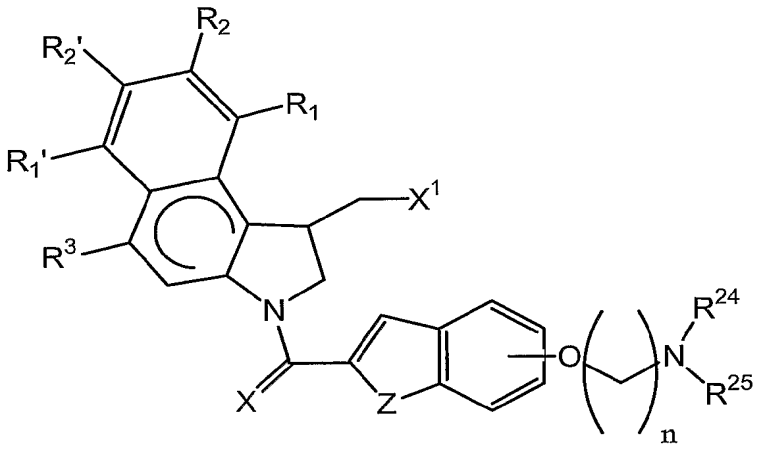
130. The compound according to any one of claims 125 to 129, wherein R^1, $R^{1'}$, R^2, and $R^{2'}$ are H.

131. The compound according to any one of claims 125 to 130, wherein X is O and Z is O.

132. The compound according to any one of claims 125 to 131, wherein the compound has the following formula:

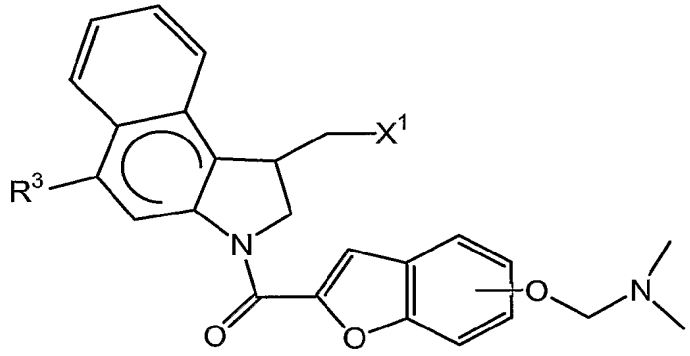


133. The compound according to any one of claims 125 to 132, wherein the compound has the following formula:

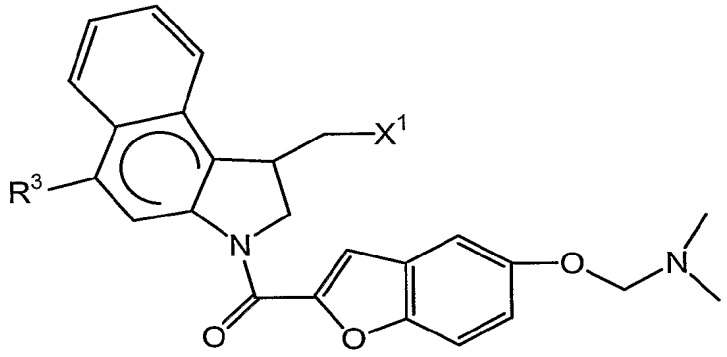


wherein X^1 is F, Cl, or Br.

134. The compound according to any one of claims 125 to 133, wherein the compound has the following formula:



135. The compound according to any one of claims 125 to 134, wherein the compound has the following formula:



136. A pharmaceutical formulation comprising a compound according to any one of claims 1 to 135 and a pharmaceutically acceptable carrier.

137. A method of killing a cell, said method comprising administering to said cell an amount of a compound according to to any one of claims 1 to 135 sufficient to kill said cell.

138. The method of claim 137, wherein the cell is a tumor cell.

139. A method of retarding or stopping the growth of a tumor in a mammalian subject, comprising administering to said subject an amount of a compound according to to any one of claims 1 to 135, sufficient to retard or stop the growth.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 June 2006 (22.06.2006)

PCT

(10) International Publication Number
WO 2006/065533 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2005/043257

(22) International Filing Date:
29 November 2005 (29.11.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/631,757 29 November 2004 (29.11.2004) US
60/673,146 19 April 2005 (19.04.2005) US

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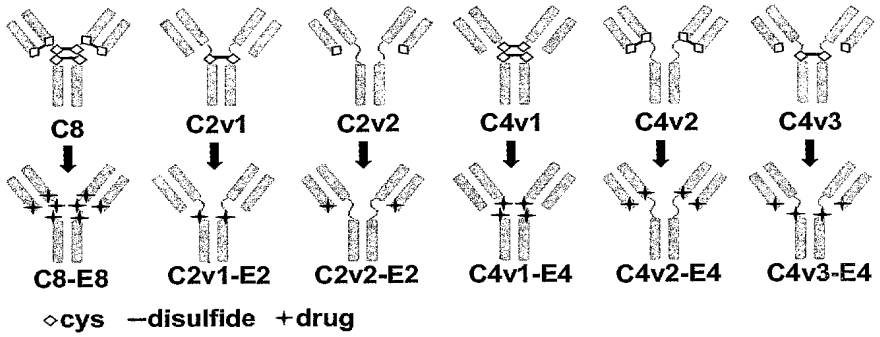
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, 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, IS, IT, LT, LU, LV, 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: ENGINEERED ANTIBODIES AND IMMUNOCONJUGATES



(57) Abstract: Antibody drug conjugates with predetermined sites and stoichiometries of drug attachment are provided. Also provided are methods of using antibody drug conjugates.

WO 2006/065533 A2

ENGINEERED ANTIBODIES AND IMMUNOCONJUGATES

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 60/631,757, filed November 29, 2004, and of U.S. Provisional Patent Application No. 60/673,146, filed April 19, 2005, each of which is hereby incorporated by reference herein in its entirety.

Background

The present invention is directed to engineered antibodies with predetermined points of attachment for an active moiety. In particular, the invention is directed to antibodies with predetermined points of attachment for active moieties by selective substitution of an amino acid residue(s) of the antibody.

The use of targeting monoclonal antibodies conjugated to radionuclides or other cytotoxic agents offers the possibility of delivering such agents directly to the tumor site, thereby limiting the exposure of normal tissues to the agents (see, e.g., Goldenberg, Semin. Nucl. Med. 19: 332 (1989)). In recent years, the potential of antibody-based therapy and its accuracy in the localization of tumor-associated antigens have been demonstrated both in the laboratory and clinical studies (see, e.g., Thorpe, TIBTECH 11:42 (1993); Goldenberg, Scientific American, Science & Medicine 1:64 (1994); Baldwin et al., U.S. Pat. Nos. 4,925,922 and 4,916,213; Young, U.S. Pat. Nos. 4,918,163 and 5,204,095; Irie et al., U.S. Pat. No. 5,196,337; Hellstrom et al., U.S. Pat. Nos. 5,134,075 and 5,171,665). In general, the use of radiolabeled antibodies or antibody fragments against tumor-associated markers has been more successful for localization of tumors than for therapy, in part because antibody uptake by the tumor is generally low, ranging from only 0.01% to 0.001% of the total dose injected (Vaughan et al., Brit. J. Radiol. 60:567 (1987)). Increasing the concentration of the radiolabel to increase the dosage to the tumor is generally counterproductive because this also increases exposure of healthy tissue to radioactivity.

Monoclonal antibodies can be conjugated to a variety of agents, other than radionuclides, to form immunoconjugates for use in diagnosis and therapy. These agents include chelates, which allow the immunoconjugate to form a stable bond with

radioisotopes, and cytotoxic agents such as toxins and chemotherapy drugs. For example, cytotoxic agents that normally would be too toxic to patients if administered in a systemic fashion can be conjugated to anti-cancer antibodies in such a manner that their toxic effects become directed only to the tumor cells bearing the target antigens. The diagnostic or therapeutic efficacy of immunoconjugates depends upon several factors. Among these factors are the molar ratio of the agent to the antibody and the binding activity of the immunoconjugate.

Researchers have found that the maximum number of agents that can be directly linked to an antibody is limited by the number of modifiable sites on the antibody molecule and the potential loss of immunoreactivity of the antibody. For example, Kulkarni et al. (Cancer Research 41:2700-2706 (1981)) have reported that there is a limit to the number of drug molecules that can be incorporated into an antibody without significantly decreasing antigen-binding activity. Kulkarni et al. found that the highest incorporation obtained for methotrexate was about ten methotrexate molecules per-molecule of antibody, and that attempts to increase the drug-antibody molar ratio over about ten decreased the yield of immunoconjugate and damaged antibody activity. Kanellos et al. (JNCI 75:319-329 (1985)) have reported similar results.

For monoclonal antibodies to function as the delivery vehicles for drugs and radionuclides, it is important to develop methods for their site-specific conjugations, with minimal perturbation of the resultant immunoreactivities. Most commonly, the conjugation of drugs and radionuclides is accomplished through covalent attachments to side chains of amino acid residues. Due to the non-site-restricted nature of these residues, it is difficult to avoid undesirable couplings at residues that lie within or are in close vicinity to the antigen binding site (ABS), leading to reduced affinity and heterogeneous antigen-binding properties. Alternatively, conjugation can be directed at sulfhydryl groups. However, direct labeling relies on the reduction of disulfide (S-S) bonds, with the possible risk of protein fragmentation. Incomplete reduction of such bonds can lead to heterogeneous patterns of attachment.

For example, early preclinical versions of the cAC10 antibody drug conjugate (directed to CD30) involved linkage of eight MMAE (monomethyl auristatin E) drug molecules to the antibody via the cysteine residues. The cysteine residues were obtained by reduction of the four interchain disulfide bonds (Doronina et al., Nat. Biotechnol.

21(7):778-84 (2003)). A recent report has described the effects of drug multiplicity on the in vivo parameters of cAC10 ADCs (Hamblett et al., Clin. Cancer Res.15: 7063-7070 (2004)). cAC10 MMAE drug conjugates with 4 drug molecules attached per antibody (designated C8-E4, where C# indicates the number of interchain cysteine residues available for conjugation and E# indicates the average number of drug molecules attached per antibody molecule) have been shown to have a greater therapeutic window than cAC10 drug conjugates with 8 drugs attached per antibody (designated C8-E8) in animal models. C8-E4 displays similar pharmacokinetic properties to cAC10 alone, while C8-E8 is cleared from circulation more rapidly (Hamblett et al., supra). These characteristics suggest that C8-E4 may be a candidate for clinical development.

The preparation of C8-E4 from cAC10 may result in low yields and heterogeneity of drug attachment, depending on the method of conjugation. One method used to obtain MMAE conjugates with less than eight drugs loaded per antibody utilizes partial reduction of cysteine residues (Hamblett et al., supra). This conjugation process results in a mixture of species with zero, two, four, six or eight drug molecules per antibody molecule (designated C8-E0, C8-E2, C8-E4, C8-E6 and C8-E8, respectively), of which approximately 30% is C8-E4. This conjugate mixture can be separated by hydrophobic interaction chromatography to obtain pure C8-E4, but this process results in a further reduction in overall yield and remaining heterogeneity because the drugs are distributed over eight possible conjugation sites. Further, reduction of the heavy to light chain disulfide bond occurs at approximately double the frequency of the heavy to heavy disulfide bonds, resulting in a 2:1 ratio of the respective C8-E4 isomers. (See, e.g., Sun, et al., Bioconjug Chem 16:1282-1290 (2005).)

Thus, there is a need for antibodies having one or more predetermined sites for stoichiometric drug attachment. These and other limitations and problems of the past are solved by the present invention.

BRIEF SUMMARY OF THE INVENTION

The invention relates to engineered antibodies and immunoconjugates. The invention provides engineered antibodies and immunoconjugates and methods of preparing such engineered antibodies and immunoconjugates. The invention also provides

pharmaceutical compositions of immunoconjugates and methods of using immunoconjugates to treat or diagnose a variety of conditions and diseases.

In one aspect, the invention provides immunoconjugates including engineered antibodies having a functionally active antigen-binding site for a target antigen, at least one interchain cysteine residue, at least one amino acid substitution of an interchain cysteine residue, and a diagnostic, preventative or therapeutic agent conjugated to at least one interchain cysteine residue. In one embodiment, the invention provides immunoconjugates having four interchain cysteine residues and four amino acid substitutions of interchain cysteine residues. In a related embodiment, the invention provides immunoconjugates having two interchain cysteine residues and six amino acid substitutions of interchain cysteine residues. In another embodiment, the invention provides immunoconjugates that are of the IgG1 or IgG4 isotype. The amino acid substitutions can be, for example, cysteine to serine amino acid substitutions of the interchain cysteine residues.

In another aspect, the invention provides immunoconjugates as described above in which a therapeutic agent is conjugated to at least one interchain cysteine residue. In one embodiment, the therapeutic agent is an auristatin or auristatin derivative. In some embodiments, the auristatin derivative is dovaline-valine-dolaisoleunine-dolaproine-phenylalanine (MMAF) or monomethyauristatin E (MMAE).

In another aspect, the invention provides immuoconjugates as described above in which a diagnostic agent is conjugated to at least one interchain cysteine residue. The diagnostic agent can be, for example, a radioactive agent, an enzyme, a fluorescent compounds or an electron transfer agent.

In another aspect, the invention provides immunoconjugates as described above in which the antibody has a functionally active antigen-binding site for a target antigen. The antibody can bind to, for example, CD20, CD30, CD33, CD40, CD70 or Lewis Y. The antibody also can bind to an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein. In other examples, the antibody binds to a microbial antigen, or viral antigen. The antibody also can be an anti-nuclear antibody, anti-ds DNA antibody, anti-ss DNA antibody, anti-cardiolipin

antibody IgM or IgG, anti-phospholipid antibody IgM or IgG, anti-SM antibody, anti-mitochondrial antibody, anti-thyroid antibody, anti-microsomal antibody, anti-thyroglobulin antibody, anti-SCL 70 antibody, anti-Jo antibody, anti-U1RNP antibody, anti-La/SSB antibody, anti-SSA antibody, anti-SSB antibody, anti-perital cells antibody, anti-histone antibody, anti-RNP antibody, anti-C ANCA antibody, anti-P ANCA antibody, anti-centromere antibody, anti-fibrillarin antibody, or anti-GBM antibody.

In another aspect, the invention provides immunoconjugates as described above in which the antibody is an antibody fragment. In one embodiment, the antibody fragment is Fab, Fab' or scFvFc.

In another aspect, the invention provides immuoconjugates of the following formula:

$$Ab_z-(A_a-W_w-Y_y-D)_p$$

or a pharmaceutically acceptable salt or solvate thereof,
wherein:
Ab is an antibody,
A is a stretcher unit,
a is 0 or 1,
each W is independently a linker unit,
w is an integer ranging from 0 to 12,
Y is a spacer unit, and
y is 0, 1 or 2,
p ranges from 1 to about 20, and
D is a diagnostic, preventative and therapeutic agent, and
z is the number of predetermined conjugation sites on the protein.

In some embodiments, the immunoconjugates are of the formula: Ab-MC-vc-PAB-MMAF, Ab-MC-vc-PAB-MMAE, Ab-MC-MMAE or Ab-MC-MMAF.

In another aspect, the invention provides pharmaceutical compositions containing the immunoconjugates described above and a pharmaceutical acceptable carrier. In an

embodiment, the immunoconjugate is formulated with a pharmaceutically acceptable parenteral vehicle. In another embodiment, the immunoconjugate is formulated in a unit dosage injectable form. In a related aspect, the invention provides an article of manufacture having an immunoconjugate conjugated to a therapeutic agent, a container, and a package insert or label indicating that the compound can be used to treat cancer characterized by the overexpression of at least one of CD20, CD30, CD33, CD40, CD70 and Lewis Y.

In another aspect, the invention provides methods of treating a variety of conditions or diseases using immunoconjugates described above that are conjugated to a therapeutic agent. In one embodiment, the methods involve killing or inhibiting the proliferation of tumor cells or cancer cells by treating tumor cells or cancer cells with an amount the immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to kill or inhibit the proliferation of the tumor cells or cancer cells. In another embodiment, the methods involve treating cancer by administering to a patient an amount of immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to treat cancer. In another embodiment, the methods involve treating an autoimmune disease by administering to a patient an amount of immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to treat the autoimmune disease. In yet another embodiment, the methods involve treating an infectious disease by administering to a patient an amount of an immunoconjugate, or a pharmaceutically acceptable salt or solvate, effective to treat the infectious disease.

In another aspect, the invention provides methods of diagnosing a variety of conditions or diseases using immunoconjugates described above that are conjugated to a diagnostic agent. In one embodiment, the methods involve diagnosing cancer by administering to a patient an effective amount of immunoconjugate that binds to an antigen overexpressed by the cancer, and detecting the immunoconjugate in the patient. In another embodiment, the methods involve diagnosing an infectious disease by administering to a patient an effective amount of the immunoconjugate that binds to a microbial or viral antigen, and detecting the immunoconjugate in the patient. In yet another embodiment, the methods involve diagnosing an autoimmune disease in a patient by administering an effective amount of immunoconjugate that binds to an antigen

associated with the autoimmune disease, and detecting the immunoconjugate in the patient.

In another aspect, the invention provides methods of preparing an immunoconjugate involving culturing a host cell expressing an engineered antibody having a functionally active antigen-binding region for a target antigen, at least one interchain cysteine residue, and at least one amino acid substitution of an interchain cysteine residue. The host cell can be transformed or transfected with an isolated nucleic acid encoding the engineered antibody. The antibody can be recovered from the cultured host cells or the culture medium, and conjugated to a diagnostic, preventative or therapeutic agent via at least one interchain cysteine residue. In an embodiment, the antibody is an intact antibody or an antigen-binding fragment. In a preferred embodiment, the antigen binding fragment is an Fab, Fab' or scFvFc.

The invention will best be understood by reference to the following detailed description of the preferred embodiment, taken in conjunction with the accompanying drawings. The discussion below is descriptive, illustrative and exemplary and is not to be taken as limiting the scope defined by any appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the design and analysis of antibody Cys→Ser variants and corresponding antibody drug conjugates (ADCs). (A) Schematic representation of antibody variants and drug conjugates highlighting the location of accessible cysteines (diamonds), inter-chain disulfide bonds (-) and subsequently conjugated drugs (+). Antibodies and ADCs are identified by their variant name (see Table 1), and loading stoichiometry with the drug, MMAE. For example, C8-E8 denotes the ADC in which all eight solvent accessible interchain cysteine residues in the cAC10 parent antibody (C8) are conjugated to MMAE (E8). (B) SDS-PAGE analysis of antibody variants under non-reducing conditions. HHLL, HH, HL, H and L indicate migration patterns for antibody heavy-light chain tetramer, heavy chain dimer, heavy-light chain dimer, heavy chain and light chain, respectively. (C) SDS-PAGE analysis of antibody variant conjugates with MMAE under reducing conditions.

Figure 2 shows titration profiles of a growth proliferation assay using antibody cysteine variants and parent cAC10 antibody conjugated to MC-vcMMAE. (A) Serial dilutions of cAC10 ADCs C2v1-E2, C4v1-E4, C4v2-E4, C6v1-E6 and C8-E4 were incubated with Karpas-299 cells for 96 hours. [H^3]-TdR was then added and its incorporation measured. (B) Karpas-299 cells were incubated with cAC10 ADCs C2v1-E2, C2v2-E2 and C8-E2 for 96 hours. Resazurin was then added and dye reduction measured.

Figure 3 shows single dose efficacy studies on SCID mice bearing Karpas-299 subcutaneous xenografts that were treated with antibody cysteine variants and parent cAC10 antibody conjugated to MC-vcMMAE. Mice were treated with a single dose of C2v1-E2and C8-E2 at 2 mg/kg (A) and C4v1-E4, C4v2-E4, and C8-E4 at 1 mg/kg (B).

Figure 4 shows plasmid map pBSSK AC10H.

Figure 5 shows plasmid map pBSSK AC10 L.

Figure 6 shows reverse phase HPLC analysis of ADCs under reducing conditions. (A) C8-E4M. (B) C8-E4. (C) C4v1-E4. (D) C4v2-E4. (See Table 1). Peaks were identified by the ratio of their absorbances at wavelengths of 248 nm and 280 nm. L-E0 and L-E1 are used to denote light chains loaded with 0 or 1 equivalents of MMAE, respectively, whereas H-E0, H-E1, H-E2 and H-E3 indicate heavy chains loaded with 0, 1, 2, or 3 equivalents of MMAE, respectively.

Figure 7 shows single dose efficacy studies on SCID mice bearing L540cy subcutaneous xenografts. Mice were treated 12 days post tumor implant with a single dose of C2v1-E2, C2v2-E2 and C8-E2 at 6 mg/kg (A) or 12 mg/kg (B). Mice were dosed with C4v1-E4, C4v2-E4, C8-E4 and C8-E4M at 3 mg/kg (C) and 6 mg/kg (D).

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art pertinent to the methods and compositions described. As used herein, the following terms and phrases have the meanings ascribed to them unless specified otherwise.

Antibody. As used herein, "antibody" refers to monoclonal antibodies, such as murine, chimeric, human, or humanized antibodies, mixtures of antibodies, as well as antigen-binding fragments thereof. Such fragments include Fab, Fab', $F(ab)_2$, and $F(ab')_2$. Antibody fragments also include isolated fragments consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, and recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker (e.g., scFv and scFvFc). In some embodiments, the antibody comprises at least one interchain cysteine residue.

Intact Antibody. An "intact" antibody is one which comprises a V_L and V_H antigen-binding variable regions as well as light chain constant domain (C_L) and heavy chain constant domains, C_H1, C_H2, C_H3, and C_H4. The constant domains may be native sequence constant domains (e.g., human native sequence constant domains) or amino acid sequence variants thereof.

Interchain Cysteine Residue: As used herein, "interchain cysteine residue" or "interchain cysteine" refer to a cysteine residue of an antibody chain that can be involved in the formation of an interchain disulfide bond with a cysteine residue of another chain of the unengineered antibody. The interchain cysteine residues are located in the C_L domain of the light chain, the C_H1 domain of the heavy chain, and in the hinge region. The number of interchain cysteine residues in an antibody can vary. For example, human IgG1, IgG2, IgG3 and IgG4 isotypes have 4, 6, 13 and 4 interchain cysteine bonds, respectively. In a specific example, by reference to antibody cAC10, the interchain cysteine thiols are located at amino acid position 214 of the light chain and at amino acid positions 220, 226 and 229 of the heavy chain, according to the numbering scheme of Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed. NIH, Bethesda, MD (1991)).

Interchain Disulfide Bond. The term "interchain disulfide bond," in the context of an antibody, refers to a disulfide bond between two heavy chains, or a heavy and a light chain.

Engineered Antibody. As used herein, an "engineered antibody" refers to a nonnaturally occurring intact antibody or antigen-binding fragment having at least one amino acid substitution of an interchain cysteine residue for another amino acid residue

(e.g., a cysteine to serine substitution), and retaining at least one unsubstituted interchain cysteine residue.

Isomer. The term "isomer" in the context of an antibody refers to an antibody having a particular pattern or order of amino acid substitutions of interchain cysteine residues. In the context of an immunoconjugate, the term "isomer" refers to an antibody having a particular pattern or order of amino acid substitutions of interchain cysteine residues and/or a particular pattern of sites of conjugation of an active moiety or moieties. An isomer of an antibody can be referred to by the nomenclature C#v#, where C# indicates the number of interchain cysteine residues available for conjugation and v# refers to a particular pattern or order of interchain cysteine residues. An isomer of an immunoconjugate can be referred to by the nomenclature C#v#-Y, where C# and v# have the same meaning as stated above and Y refers to the average number of diagnostic, preventative or therapeutic agents attached per antibody molecule.

Fully-Loaded. The term "fully-loaded" refers to an antibody in which the predetermined points of conjugation of a particular type and/or of similar reactivity are conjugated to an active moiety, resulting in a homogeneous population of the immunoconjugate (C# = Y).

Partially-Loaded. The term "partially-loaded" refers to an antibody in which only some of the predetermined points of conjugation of a particular type and/or of a similar reactivity are conjugated to an active moiety, resulting in formation of a certain isomer or isomers of the immunoconjugate (C# > Y).

Diagnostic, Preventative or Therapeutic Agent. As used herein, a "diagnostic, preventative or therapeutic agent" is an active moiety such as a macromolecule, molecule or atom which is conjugated to an antibody to produce an immunoconjugate which is useful for diagnosis, prevention and/or for therapy. Examples of diagnostic, preventative or therapeutic agents include drugs, toxins, and detectable labels.

Immunoconjugate. As used herein, an "immunoconjugate" is a molecule comprising an antibody conjugated directly or indirectly to at least one diagnostic, preventative and/or therapeutic agent, or a chelating agent that binds the diagnostic, preventative and/or therapeutic agent. An immunoconjugate retains the immunoreactivity of the antibody, e.g., the antibody has approximately the same, or only slightly reduced,

ability to bind the antigen after conjugation as before conjugation. As used herein, an immunoconjugate is also referred to as an antibody drug conjugate (ADC).

Functionally Active. The term "functionally active,"in the context of an antibody means the antibody immunospecifically binds to a target antigen.

Isolated. The term "isolated," in the context of a molecule or macromolecule (e.g., an antibody or nucleic acid) is one which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with the desired use (e.g., diagnostic or therapeutic) of the molecule, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In some embodiments, an isolated molecule or macromolecule will be purified (1) to greater than 95%, or greater than 99%, by weight of the molecule or macromolecule as determined by, for example, the Lowry or Bradford methods, (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 sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions determined by, for example, Coomassie blue or, preferably, silver staining methods. Isolated molecules and macromolecules include the molecule and macromolecule in situ within recombinant cells since at least one component of the molecules' and macromolecules' natural environment will not be present. Ordinarily, however, isolated molecules and macromolecules will be prepared by at least one purification step.

Structural gene. As used herein, a "structural gene" is a DNA molecule having a sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Promoter. As used herein, a "promoter" is a sequence of a nucleic acid that directs the transcription of a structural gene to produce mRNA. Typically, a promoter is located in the 5' region of a gene, proximal to the start codon of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Enhancer. As used herein, an "enhancer" is a promoter element that can increase the efficiency with which a particular gene is transcribed into mRNA, irrespective of the distance or orientation of the enhancer relative to the start site of transcription.

Complementary DNA (cDNA). As used herein, "complementary DNA" is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to a portion(s) of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complement.

Expression. As used herein, "expression" is the process by which a polypeptide is produced from a structural gene or cDNA molecule. The process involves transcription of the coding region into mRNA and the translation of the mRNA into a polypeptide(s).

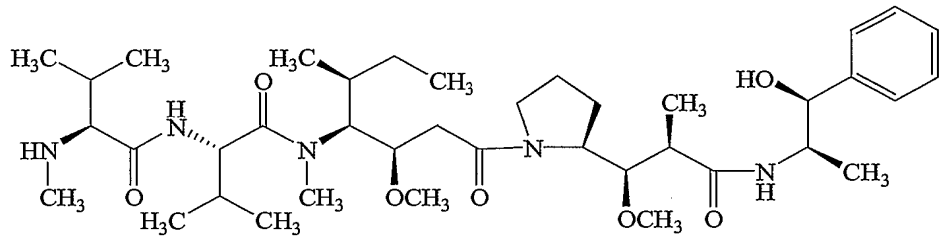
Cloning vector. As used herein, a "cloning vector" is a DNA molecule, such as a plasmid, cosmid, or bacteriophage, which has the capability of replicating autonomously in a host cell and which is used to transform cells for gene manipulation. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences may be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene which is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

Expression vector. As used herein, an "expression vector" is a DNA molecule comprising a heterologous structural gene or cDNA encoding a foreign protein which provides for the expression of the foreign protein in a recombinant host. Typically, the expression of the heterologous gene is placed under the control of (i.e., operably linked to) certain regulatory sequences such as promoter and/or enhancer sequences. Promoter sequences may be either constitutive or inducible.

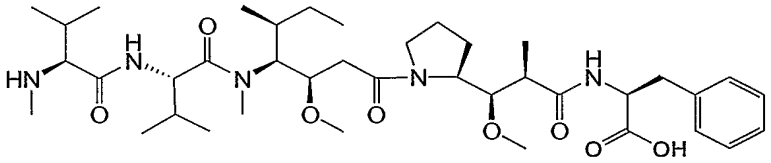
Recombinant Host. A "recombinant host" may be any prokaryotic or eukaryotic cell for expression of a heterologous (foreign) protein. In some embodiments, the recombinant host contains a cloning vector or an expression vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically

engineered to contain a nucleic acid encoding the heterologous protein in the chromosome or genome of the host cell. For examples of suitable hosts, see, e.g., Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989); Sambrook et al., Molecular Cloning, A Laboratory Manual, Third Edition, Cold Spring Harbor Publish., Cold Spring Harbor, New York (2001); and Ausubel et al., Current Protocols in Molecular Biology, 4th ed., John Wiley and Sons, New York (1999); all of which are incorporated by reference herein.

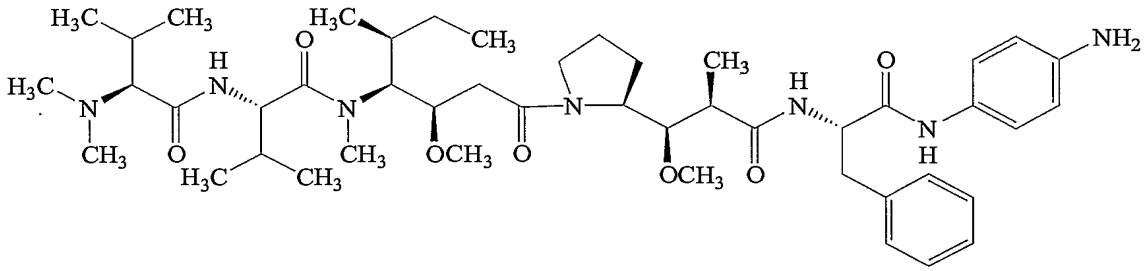
MMAE. The abbreviation "MMAE" refers to monomethyl auristatin E:



MMAF. The abbreviation "MMAF" refers to dovaline-valine-dolaisoleucine-dolaproline-phenylalanine:



AFP. The abbreviation "AFP" refers to dimethylvaline-valine-dolaisoleucine-dolaproline-phenylalanine-p-phenylenediamine:



AEB. The abbreviation "AEB" refers to an ester produced by reacting auristatin E with paraacetyl benzoic acid.

AEVB. The abbreviation "AEVB" refers to an ester produced by reacting auristatin E with benzoylvaleric acid.

Patient. A "patient" includes, but is not limited to, a human, rat, mouse, guinea pig, monkey, pig, goat, cow, horse, dog, cat, bird and fowl.

Effective Amount. The term "effective amount" refers to an amount of a diagnostic, preventative or therapeutic agent sufficient for diagnosis, prevention or treatment of a disease or disorder in a mammal.

Therapeutically Effective Amount. The term "therapeutically effective amount" refers to an amount of a drug , toxin or other molecule effective to prevent or treat a disease or disorder in a mammal. In the case of cancer, the therapeutically effective amount may reduce the number of cancer cells; reduce the tumor size; inhibit (i.e., slow to some extent and preferably stop) cancer cell infiltration into peripheral organs; inhibit (i.e., slow to some extent and preferably stop) tumor metastasis; inhibit, to some extent, tumor growth; and/or relieve to some extent one or more of the symptoms associated with the cancer. To the extent the drug, toxin or other molecule may prevent growth and/or kill existing cancer cells, it may be cytostatic and/or cytotoxic. For cancer therapy, efficacy can, for example, be measured by assessing the time to disease progression (TTP) and/or determining the response rate (RR).

The phrase "pharmaceutically acceptable salt," as used herein, refers to pharmaceutically acceptable organic or inorganic salts of a molecule or macromolecule. Acid addition salts can be formed with amino groups. Exemplary salts include, but are not limited, to sulfate, citrate, acetate, oxalate, chloride, bromide, iodide, nitrate, bisulfate, phosphate, acid phosphate, isonicotinate, lactate, salicylate, acid citrate, tartrate, oleate, tannate, pantothenate, bitartrate, ascorbate, succinate, maleate, gentisinate, fumarate, gluconate, glucuronate, saccharate, formate, benzoate, glutamate, methanesulfonate, ethanesulfonate, benzenesulfonate, p-toluenesulfonate, and pamoate (i.e., 1,1' methylene bis -(2-hydroxy 3-naphthoate)) salts. A pharmaceutically acceptable salt may involve the inclusion of another molecule such as an acetate ion, a succinate ion or other counterion. The counterion may be any organic or inorganic moiety that stabilizes the charge on the parent compound. Furthermore, a pharmaceutically acceptable salt may have more than one charged atom in its structure. Where multiple charged atoms are part of the pharmaceutically acceptable salt, the salt can have multiple counter ions. Hence, a pharmaceutically acceptable salt can have one or more charged atoms and/or one or more counterion.

"Pharmaceutically acceptable solvate" or "solvate" refer to an association of one or more solvent molecules and a molecule or macromolecule. Examples of solvents that form pharmaceutically acceptable solvates include, but are not limited to, water, isopropanol, ethanol, methanol, DMSO, ethyl acetate, acetic acid, and ethanolamine.

DETAILED DESCRIPTION

The present invention provides engineered antibodies and immunoconjugates, and methods of preparing such antibodies and immunoconjugates. The engineered antibodies have at least one predetermined site for conjugation to an active moiety, such as a diagnostic, preventative or therapeutic agent. In some aspects, the engineered antibodies can be stoichiometrically conjugated to a diagnostic, preventative or therapeutic agent to form immunoconjugates with predetermined average loading of the agent. The immunoconjugates can be used therapeutically, diagnostically (e.g., in vitro or in vivo), for in vivo imaging, and for other uses. For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

Engineered Antibodies

In one aspect, engineered antibodies are provided. An engineered antibody has an amino acid substitution of at least one interchain cysteine residue, while retaining at least one interchain cysteine residue for conjugation to a diagnostic, preventative or therapeutic agent.

In some embodiments, the antibody is an intact antibody. The antibody can be, for example, of the IgG, IgA, IgM, IgD or IgE class, and within these classes, various subclasses, such as an IgG1, IgG2, IgG3, IgG4, IgA1 or IgA2 isotypes. For example, in some embodiments, the antibody can be an IgG, such as an IgG1, IgG2, IgG3 or IgG4.

In some embodiments, the engineered antibody comprises at least one amino acid substitution replacing an interchain cysteine residue with another amino acid. The interchain cysteine residue can be involved in the formation of an interchain disulfide bond between light and heavy chains and/or between heavy chains. Thus, the amino acid substitution can be in the interchain cysteine residues in the C_L domain of the light chain, the C_H1 domain of the heavy chain, and/or in the hinge region. For example, with reference to antibody cAC10, the interchain cysteine residues are at amino acid positions

214 of the light chain and at amino acid positions 220 (C_H1) and 226 and 229 (hinge region) in the heavy chain in the numbering scheme of Kabat (Kabat et al., Sequences of Proteins of Immunological Interest, 5th ed. NIH, Bethesda, MD (1991)). One or more of these interchain cysteine residues in cAC10 can be substituted.

In some embodiments, the amino acid substitution is a serine for a cysteine residue. In some embodiments, the amino acid substitution introduces is a serine or threonine residue. In some embodiments, the amino acid substitution introduces is a serine, threonine or glycine residue. In some embodiments, the amino acid substitution introduces a neutral (e.g., serine, threonine or glycine) or hydrophilic (e.g., methionine, alanine, valine, leucine or isoleucine) residue. In some embodiments, the amino acid substitution introduces a natural amino acid, other than a cysteine residue.

The engineered antibody retains at least one unsubstituted interchain cysteine residue for conjugation to an active moiety. The number of retained intercysteine residues in an engineered antibody is greater than zero but less than the total number of interchain cysteine residues in the parent (non-engineered) antibody. Thus, in some embodiments, the engineered antibody has at least one, at least two, at least three, at least four, at least five, at least six or at least seven interchain cysteine residues. In typical embodiments, the engineered antibody has an even integral number of interchain cysteine residues (e.g., at least two, four, six or eight reactive sites). In some embodiments, the engineered antibody has less than eight interchain cysteine residues.

In a typical embodiment, the interchain cysteine residues are substituted in a pairwise manner, in which both cysteine residues involved in the formation of an interchain disulfide bond are substituted. (Such interchain cysteine residues can be referred to as "complementary" interchain cysteine residues.) For example, if the C_L interchain cysteine residue(s) are substituted, the complementary C_H1 interchain cysteine residue(s) might also be substituted. In another example, each pair of the interchain cysteine residues in the hinge region can be substituted or remain unsubstituted in a pairwise manner. In other embodiments, an interchain cysteine residue can be substituted while the complementary residue can remain unsubstituted.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each

having an amino acid substitution of the C_H1 interchain cysteine residue and retaining the interchain cysteine residues in the hinge region. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of at least one of the interchain cysteine residues in the hinge region. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the remaining interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and having amino acid substitutions of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of such an engineered antibody has active moieties conjugated to the C_L interchain cysteine residues and heavy chains C_H1 interchain cysteine residues.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and having amino acid substitutions of at least one but less than all of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of such an engineered antibody has active moieties conjugated to the C_L interchain cysteine residues, to heavy chains C_H1 interchain cysteine residues and to the remaining interchain cysteine residues.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of at least one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_L interchain cysteines and to the remaining interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid

substitution of the C_H1 interchain cysteine residue and an amino acid substitution of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_L interchain cysteine residues.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine residue and the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_H1 interchain cysteine residues and to the interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine residue and having an amino acid substitution of at least one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_H1 interchain cysteine residues and to the remaining interchain cysteine residues of the hinge region.

In some embodiments, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine residue and having an amino acid substitution of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has active moieties conjugated to the C_H1 interchain cysteine residues.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and retaining the interchain cysteine residues in the hinge region. In a related embodiment, an immunoconjugate of the engineered antibody has four active moieties conjugated to the interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and having amino acid substitutions of both hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of such an engineered antibody has four active moieties conjugated to the C_L interchain cysteine residues and heavy chains C_H1 interchain cysteine residues.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has four active moieties conjugated to the C_L interchain cysteines and to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and a substitution of one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has two active moieties conjugated to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and an amino acid substitution of both hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has two active moieties conjugated to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having the C_H1 interchain cysteine

residue and an amino acid substitution of one of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has six active moieties conjugated to the C_L interchain cysteine residues and to the remaining interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having the C_L interchain cysteine residue and heavy chains each having an amino acid substitution of the C_H1 interchain cysteine residue and retaining both of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has six active moieties conjugated to the C_L interchain cysteine residues and to the interchain cysteine residues of the hinge region.

In an exemplary embodiment where the parent antibody has eight interchain cysteine residues, the engineered antibody comprises light chains each having an amino acid substitution of the C_L interchain cysteine residue and heavy chains each retaining the C_H1 interchain cysteine residue and both of the hinge region interchain cysteine residues. In a related embodiment, an immunoconjugate of the engineered antibody has six active moieties conjugated to the C_H1 interchain cysteine residues and to the interchain cysteine residues of the hinge region.

The antibody also can be an antigen-binding antibody fragment such as, for example, a Fab, a F(ab'), a $F(ab')_2$, a Fd chain, a single-chain Fv (e.g., scFv and scFvFc), a single-chain antibody, a disulfide-linked Fv (sdFv), a fragment comprising either a V_L or V_H domain, a minibody, a maxibody, an $F(ab')_3$, or fragments produced by a Fab expression library. Antigen-binding antibody fragments, including single-chain antibodies, can comprise the variable region(s) alone or in combination with the entirety or a portion of the following: hinge region, C_H1, C_H2, C_H3, C_H4 and/or C_L domains. Also, antigen-binding fragments can comprise any combination of variable region(s) with a hinge region, C_H1, C_H2, C_H3, C_H4 and/or C_L domains. See also Holliger and Hudson, Nat. Biotechnol. 23:1126-1136 (2005), the disclosure of which is incorporated by reference herein.

In some embodiments, an antibody fragment comprises at least one domain, or part of a domain, that includes at least one interchain cysteine residue. For example, the

antibody fragment can include a hinge region, a C_L and C_H1 domains, C_L and C_H1 domains and a hinge region, or the like.

The antibody fragment can be of any suitable antibody class (e.g., IgG, IgA, IgM, IgD and IgE) and subclass (e.g., IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2).

Typically, the antibodies are human, rodent (e.g., mouse, rat or hamster), donkey, sheep, rabbit, goat, guinea pig, camelid, horse, or chicken. As used herein, "human" antibodies include antibodies having the amino acid sequence of a human immunoglobulin and include antibodies isolated from human immunoglobulin libraries, from human B cells, or from animals transgenic for one or more human immunoglobulins, as described infra and, for example in Reichert et al. (Nat. Biotechnol. 23:1073-8 (2005)) and in U.S. Patent Nos. 5,939,598 and 6,111,166. The antibodies may be monospecific, bispecific, trispecific, or of greater multispecificity.

The antibody is typically a monoclonal antibody but also can be a mixture of monoclonal antibodies. When the subject is a human subject, the antibody may be obtained by immunizing any animal capable of mounting a usable immune response to the antigen. The animal may be a mouse, rat, goat, sheep, rabbit or other suitable experimental animal. The antigen may be presented in the form of a naturally occurring immunogen, or a synthetic immunogenic conjugate of a hapten and an immunogenic carrier. The antibody producing cells of the immunized animal may be fused with "immortal" or "immortalized" human or animal cells to obtain a hybridoma which produces the antibody. If desired, the genes encoding one or more of the immunoglobulin chains may be cloned so that the antibody may be produced in different host cells, and if desired, the genes may be mutated so as to alter the sequence and hence the immunological characteristics of the antibody produced. (See also Teng et al. Proc. Natl. Acad. Sci. USA. 80:7308-7312 (1983); Kozbor et al., Immunology Today 4:72-79 (1983); and Olsson et al., Meth. Enzymol. 92:3-16 (1982)). Human monoclonal antibodies may be made by any of numerous techniques known in the art, such as phage display (see, e.g., Hoogenboom, Nat. Biotechnol. 23:1105-16 (2005); transgenic mice expressing human immunoglobulin genes (see, e.g., Lonberg, Nat. Biotechnol. 23:1117-25 (2005)); ribosome-, mRNA- and yeast-display libraries (see, e.g., Hoogenboom, supra), and human hybridomas from patients (Brändlein et al., Histol. Histopathol. 19:897-905 (2004); and Illert et al., Oncol. Rep. 13:765-70 (2005)), and/or single-antigen selected lymphocytes

(see, e.g., Lagerkvist et al., Biotechniques 18:862-9 (1995); and Babcook et al., Proc. Natl. Acad. Sci. USA 93:7843-8 (1996)).

The antibody can be, for example, a murine, a chimeric, humanized, or fully human antibody produced by techniques well-known to one of skill in the art. Recombinant antibodies, such as chimeric and humanized monoclonal antibodies, comprising both human and non-human portions, which can be made using standard recombinant DNA techniques, are useful antibodies. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable region derived from a murine monoclonal and human immunoglobulin constant regions. (See, e.g., Cabilly et al., U.S. Patent No. 4,816,567; and Boss et al., U.S. Patent No. 4,816,397, which are incorporated herein by reference in their entirety.) In some embodiments, the antibody light chain constant region domain is not chimeric. In some embodiments, the antibody heavy chain constant region is not chimeric. In this context, "chimeric" refers to a constant region or constant region domain composed of portions from two different species.

The antibody can also be a bispecific antibody. Methods for making bispecific antibodies are known in the art. Traditional production of full-length bispecific antibodies is based on the coexpression of two immunoglobulin heavy chain-light chain pairs, where the two chains have different specificities (Milstein et al., Nature 305:537-539 (1983)). For further details for generating bispecific antibodies see, for example, Suresh et al., Methods in Enzymology 121:210 (1986); Rodrigues et al., J. Immunology 151:6954-6961 (1993); Carter et al., Bio/Technology 10:163-167 (1992); Carter et al., J. Hematotherapy 4:463-470 (1995); Merchant et al., Nature Biotechnology 16:677-681 (1998)). Using such techniques, bispecific antibodies can be prepared for use in the treatment or prevention of disease. Bifunctional antibodies are also described in European Patent Publication No. EPA 0 105 360. Hybrid or bifunctional antibodies can be derived either biologically, i.e., by cell fusion techniques, or chemically, especially with cross-linking agents or disulfide-bridge forming reagents, and may comprise whole antibodies or fragments thereof. Methods for obtaining such hybrid antibodies are disclosed for example, in International Publication WO 83/03679, and European Patent Publication No. EPA 0 217 577, both of which are incorporated herein by reference.

In some embodiments, the antibody constant domains have effector function. The term "antibody effector function(s)," or AEC, as used herein refers to a function contributed by an Fc domain(s) of an Ig. Such function can be effected by, for example, binding of an Fc effector domain(s) to an Fc receptor on an immune cell with phagocytic or lytic activity or by binding of an Fc effector domain(s) to components of the complement system. The effector function can be, for example, "antibody-dependent cellular cytotoxicity" or ADCC, "antibody-dependent cellular phagocytosis" or ADCP, "complement-dependent cytotoxicity" or CDC. In other embodiments, the constant domain(s) lacks one or more effector functions.

The antibodies may be directed against an antigen of interest, such as diagnostic preventative and/or therapeutic interest. For example, the antigen can be one associated with infectious pathogens (such as but not limited to viruses, bacteria, fungi, and protozoa), parasites, tumor cells, or particular medical conditions. In the case of a tumor-associated antigen (TAA), the cancer may be of the immune system, lung, colon, rectum, breast, ovary, prostate gland, head, neck, bone, or any other anatomical location. In some embodiments, the antigen is CD2, CD20, CD22, CD30, CD33, CD38, CD40, CD52, CD70, HER2, EGFR, VEGF, CEA, HLA-DR, HLA-Dr10, CA125, CA15-3, CA19-9, L6, Lewis X, Lewis Y, alpha fetoprotein, CA 242, placental alkaline phosphatase, prostate specific membrane antigen, prostate specific antigen, prostatic acid phosphatase, epidermal growth factor, MAGE-1, MAGE-2, MAGE-3, MAGE-4, anti-transferrin receptor, p97, MUC1, gp100, MART1, IL-2 receptor, human chorionic gonadotropin, mucin, P21, MPG, and Neu oncogene product.

Some specific useful antibodies include, but are not limited to, BR96 mAb (Trail et al., Science 261:212-215 (1993)), BR64 (Trail et al., Cancer Research 57:100-105 (1997)), mAbs against the CD 40 antigen, such as S2C6 mAb (Francisco et al., Cancer Res. 60:3225-3231 (2000)), and mAbs against the CD30 antigen, such as AC10 (Bowen et al., J. Immunol. 151:5896-5906 (1993)). Many other internalizing antibodies that bind to tumor specific antigens can be used, and have been reviewed (see, e.g., Franke et al., Cancer Biother. Radiopharm. 15:459-76 (2000); Murray, Semin Oncol. 27:64-70 (2000); Breitling et al., Recombinant Antibodies, John Wiley, and Sons, New York, 1998). The disclosures of these references are incorporated by reference herein.

In some embodiments, the antigen is a "tumor-specific antigen." A "tumor-specific antigen" as used herein refers to an antigen characteristic of a particular tumor, or strongly correlated with such a tumor. However, tumor-specific antigens are not necessarily unique to tumor tissue, i.e., antibodies to tumor-specific antigens may cross-react with antigens of normal tissue. Where a tumor-specific antigen is not unique to tumor cells, it frequently occurs that, as a practical matter, antibodies binding to tumor-specific antigens are sufficiently specific to tumor cells to carry out the desired procedures without unwarranted risk or interference due to cross-reactions. Many factors contribute to this practical specificity. For example, the amount of antigen on the tumor cell may greatly exceed the amount of the cross-reactive antigen found on normal cells, or the antigen on the tumor cells may be more effectively presented. Therefore the term "tumor-specific antigen" relates herein to a specificity of practical utility, and is not intended to denote absolute specificity or to imply an antigen is unique to the tumor.

The nucleotide sequence encoding antibodies that are immunospecific for tumor associated or tumor specific antigens can be obtained, e.g., from the GenBank database or a database like it, commercial sources, literature publications, or by routine cloning and sequencing.

In some embodiments, the antibodies are directed against an antigen for the diagnosis, treatment or prevention of an autoimmune disease. Antibodies immunospecific for an antigen of a cell that is responsible for producing autoimmune antibodies can be obtained from the GenBank database or a database like it, a commercial or other source or produced by any method known to one of skill in the art such as, e.g., chemical synthesis or recombinant expression techniques.

In some embodiments, the antibody is an anti-nuclear antibody; anti-ds DNA; anti-ss DNA, anti-cardiolipin antibody IgM, IgG; anti-phospholipid antibody IgM, IgG; anti-SM antibody; anti-mitochondrial antibody; thyroid antibody; microsomal antibody; thyroglobulin antibody; anti-SCL 70; anti-Jo; anti-U1RNP; anti-La/SSB; anti-SSA; anti-SSB; anti-perital cells antibody; anti-histones; anti-RNP; anti-C ANCA; anti-P ANCA; anti-centromere; anti-fibrillarin, or an anti-GBM antibody.

In some embodiments, the antibody can bind to a receptor or a receptor complex expressed on a target cell (e.g., an activated lymphocyte). The receptor or receptor

complex can comprise an immunoglobulin gene superfamily member, a TNF receptor superfamily member, an integrin, a cytokine receptor, a chemokine receptor, a major histocompatibility protein, a lectin, or a complement control protein. Non-limiting examples of suitable immunoglobulin superfamily members are CD2, CD3, CD4, CD8, CD19, CD22, CD28, CD79, CD90, CD152/CTLA 4, PD 1, and ICOS. Non-limiting examples of suitable TNF receptor superfamily members are CD27, CD40, CD95/Fas, CD134/OX40, CD137/4 1BB, TNF R1, TNF R2, RANK, TACI, BCMA, osteoprotegerin, Apo2/TRAIL R1, TRAIL R2, TRAIL R3, TRAIL R4, and APO 3. Non-limiting examples of suitable integrins are CD11a, CD11b, CD11c, CD18, CD29, CD41, CD49a, CD49b, CD49c, CD49d, CD49e, CD49f, CD103, and CD104. Non-limiting examples of suitable lectins are C type, S type, and I type lectin. In other embodiments, the receptor is CD70.

In some embodiments, the antibody is immunospecific for a viral or a microbial antigen. As used herein, the term "viral antigen" includes, but is not limited to, any viral peptide, polypeptide protein (e.g., HIV gp120, HIV nef, RSV F glycoprotein, influenza virus neuraminidase, influenza virus hemagglutinin, HTLV tax, herpes simplex virus glycoprotein (e.g., gB, gC, gD, and gE) and hepatitis B surface antigen) that is capable of eliciting an immune response. As used herein, the term "microbial antigen" includes, but is not limited to, any microbial peptide, polypeptide, protein, saccharide, polysaccharide, or lipid molecule (e.g., a bacterial, fungi, pathogenic protozoa, or yeast polypeptide including, e.g., LPS and capsular polysaccharide 5/8) that is capable of eliciting an immune response.

Antibodies immunospecific for a viral or microbial antigen can be obtained commercially, for example, from BD Biosciences (San Francisco, CA), Chemicon International, Inc. (Temecula, CA), or Vector Laboratories, Inc. (Burlingame, CA) or produced by any method known to one of skill in the art such as, e.g., chemical synthesis or recombinant expression techniques. The nucleotide sequence encoding antibodies that are immunospecific for a viral or microbial antigen can be obtained, e.g., from the GenBank database or a database like it, literature publications, or by routine cloning and sequencing.

Examples of antibodies available useful for the diagnosis or treatment of viral infection or microbial infection include, but are not limited to, SYNAGIS (MedImmune, Inc., MD) which is a humanized anti-respiratory syncytial virus (RSV) monoclonal

antibody useful for the treatment of patients with RSV infection; PRO542 (Progenics Pharmaceuticals, Inc., NY) which is a CD4 fusion antibody useful for the treatment of HIV infection; OSTAVIR (Protein Design Labs, Inc., CA) which is a human antibody useful for the treatment of hepatitis B virus; PROTOVIR (Protein Design Labs, Inc., CA) which is a humanized IgG1 antibody useful for the treatment of cytomegalovirus (CMV); and anti-LPS antibodies.

Other antibodies include, but are not limited to, antibodies against the antigens from pathogenic strains of bacteria (e.g., Streptococcus pyogenes, Streptococcus pneumoniae, Neisseria gonorrheae, Neisseria meningitidis, Corynebacterium diphtheriae, Clostridium botulinum, Clostridium perfringens, Clostridium tetani, Hemophilus influenzae, Klebsiella pneumoniae, Klebsiella ozaenas, Klebsiella rhinoscleromotis, Staphylococc aureus, Vibrio colerae, Escherichia coli, Pseudomonas aeruginosa, Campylobacter (Vibrio) fetus, Aeromonas hydrophila, Bacillus cereus, Edwardsiella tarda, Yersinia enterocolitica, Yersinia pestis, Yersinia pseudotuberculosis, Shigella dysenteriae, Shigella flexneri, Shigella sonnei, Salmonella typhimurium, Treponema pallidum, Treponema pertenue, Treponema carateneum, Borrelia vincentii, Borrelia burgdorferi, Leptospira icterohemorrhagiae, Mycobacterium tuberculosis, Pneumocystis carinii, Francisella tularensis, Brucella abortus, Brucella suis, Brucella melitensis, Mycoplasma spp., Rickettsia prowazeki, Rickettsia tsutsugumushi, Chlamydia spp.); pathogenic fungi (e.g., Coccidioides immitis, Aspergillus fumigatus, Candida albicans, Blastomyces dermatitidis, Cryptococcus neoformans, Histoplasma capsulatum); protozoa (Entomoeba histolytica, Toxoplasma gondii, Trichomonas tenas, Trichomonas hominis, Trichomonas vaginalis, Tryoanosoma gambiense, Trypanosoma rhodesiense, Trypanosoma cruzi, Leishmania donovani, Leishmania tropica, Leishmania braziliensis, Pneumocystis pneumonia, Plasmodium vivax, Plasmodium falciparum, Plasmodium malaria); or Helminiths (Enterobius vermicularis, Trichuris trichiura, Ascaris lumbricoides, Trichinella spiralis, Strongyloides stercoralis, Schistosoma japonicum, Schistosoma mansoni, Schistosoma haematobium, and hookworms).

Other antibodies include, but are not limited to, antibodies against antigens of pathogenic viruses, including as examples and not by limitation: Poxviridae, Herpesviridae, Herpes Simplex virus 1, Herpes Simplex virus 2, Adenoviridae, Papovaviridae, Enteroviridae, Picornaviridae, Parvoviridae, Reoviridae, Retroviridae,

influenza viruses, parainfluenza viruses, mumps, measles, respiratory syncytial virus, rubella, Arboviridae, Rhabdoviridae, Arenaviridae, Hepatitis A virus, Hepatitis B virus, Hepatitis C virus, Hepatitis E virus, Non A/Non B Hepatitis virus, Rhinoviridae, Coronaviridae, Rotoviridae, and Human Immunodeficiency Virus.

Methods for Introducing an Amino Acid Substitution into an Antibody by Altering the Nucleic Acid Sequence Encoding the Protein

An amino acid substitution can be introduced into a nucleic acid sequence encoding an antibody by any suitable method. Such methods include polymerase chain reaction-based mutagenesis, site-directed mutagenesis, gene synthesis using the polymerase chain reaction with synthetic DNA oligomers, and nucleic acid synthesis followed by ligation of the synthetic DNA into an expression vector, comprising other portions of the heavy and/or light chain, as applicable. (See also Sambrook et al. and Ausubel et al., supra)

A nucleotide sequence encoding an antibody can be obtained, for example, from the GenBank database or a similar database, literature publications, or by routine cloning and sequencing. Examples of some methods that can be used for directed mutagenesis are as follows: oligonucleotide directed mutagenesis with M13 DNA, oligonucleotide directed mutagenesis with plasmid DNA, and PCR-amplified oligonucleotide directed mutagenesis. (See, e.g., Glick et al., Molecular Biotechnology: Principles and Applications of Recombinant DNA, Second Edition, ASM Press, pp. 171-182 (1998). An example of mutagenesis and cloning is described in Example 1.

Detailed protocols for oligonucleotide-directed mutagenesis and related techniques for mutagenesis of cloned DNA are well-known (see, e.g., Zoller and Smith, Nucleic Acids Res. 10:6487-6500 (1982); see also Sambrook et al., supra; and Ausubel et al., supra).

In some embodiments, the amino acid substitution is a serine for a cysteine residue. In some embodiments, the amino acid substitution introduces is a serine or threonine residue. In some embodiments, the amino acid substitution introduces a neutral (e.g., serine, threonine or glycine) or hydrophilic (e.g., methionine, alanine, valine, leucine or isoleucine) residue. In some embodiments, the amino acid substitution introduces a natural amino acid, other than a cysteine residue.

Although the present invention provides methods for introducing an amino acid substitution of an interchain cysteine residue (e.g., a cysteine to serine substitution) into an

antibody or antibody fragment, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that it is possible to introduce/remove other amino acids for conjugation, such as lysine residues, at other positions of the antibody or antibody fragment. Also, a sulfhydryl group(s) can also be recombinantly introduced into an antibody at an amino acid other than an interchain cysteine residue. Suitable alternative mutagenesis sites for conjugation can be identified using molecular modeling techniques that are well-known to those of skill in the art. See, for example, Lesk et al., "Antibody Structure and Structural Predictions Useful in Guiding Antibody Engineering," in Antibody Engineering: A Practical Guide, C. Borrebaeck (ed.), W. H. Freeman and Company, pp. 1-38 (1992); Cheetham, "Engineering Antibody Affinity," Antibody Engineering: A Practical Guide (supra) at pp. 39-67. See generally Sambrook et al., Molecular Cloning, A Laboratory Manual, 3rd ed., Cold Spring Harbor Publish., Cold Spring Harbor, New York (2001); Ausubel et al., Current Protocols in Molecular Biology, 4th ed., John Wiley and Sons, New York (1999) (all of which are incorporated by reference herein), for methods for site-directed mutagenesis.

Methods for Expressing and Isolating the Protein Product of an Engineered Antibody DNA Sequence

A. Methods for Expressing an Engineered Antibody

After altering the nucleotide sequence, the nucleic acid is inserted into a cloning vector for further analysis, such as confirmation of the nucleic acid sequence. To express the polypeptide encoded by the nucleic acid, the nucleic acid can be operably linked to regulatory sequences controlling transcriptional expression in an expression vector, then introduced into a prokaryotic or eukaryotic host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors may include translational regulatory sequences and/or a marker gene which is suitable for selection of cells that contain the expression vector.

Promoters for expression in a prokaryotic host can be repressible, constitutive, or inducible. Suitable promoters are well-known to those of skill in the art and include, for example, promoters for T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the trp, recA, heat shock, and lacZ promoters of E. coli, the alpha-amylase and the $sigma_{28}$ -specific promoters of B. subtilis, the promoters of the bacteriophages of Bacillus, Streptomyces promoters, the int promoter of bacteriophage

lambda, the bla promoter of the beta-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, J. Ind. Microbiol. 1:277-282 (1987); Watson et al., Molecular Biology Of The Gene, Fourth Edition, Benjamin Cummins (1987); Ausubel et al., supra; and Sambrook et al., supra.

In some embodiments, the prokaryotic host is E. coli. Suitable strains of E. coli include, for example, Y1088, Y1089, CSH18, ER1451 and ER1647 (see, e.g., Brown (Ed.), Molecular Biology Labfax, Academic Press (1991)). An alternative host is Bacillus subtilus, including such strains as BR151, YB886, MI119, MI120 and B170 (see, e.g., Hardy, "Bacillus Cloning Methods," in DNA Cloning: A Practical Approach, Glover (Ed.), IRL Press (1985)).

Methods for producing antibody fragments in E. coli are well-known to those in the art. See, for example, Huse, "Combinatorial Antibody Expression Libraries in Filamentous Phage," in Antibody Engineering: A Practical Guide, C. Borrebaeck (Ed.), W. H. Freeman and Company, pp. 103-120 (1992); Ward, "Expression and Purification of Antibody Fragments Using Escherichia coli as a Host," id. at pp. 121-138 (1992). Fv fragments can also be produced by methods known in the art. See, e.g., id. See also Whitlow et al., "Single-Chain Fv Proteins and their Fusion Proteins," in New Techniques In Antibody Generation, Methods 2(2) (1991). Moreover, certain expression systems for cloning antibodies in prokaryotic cells are commercially available.

In some embodiments, the nucleic acid sequence is expressed in eukaryotic cells, and especially mammalian, insect, and yeast cells. In one embodiment, the eukaryotic host is a mammalian cell. Mammalian cells provide post-translational modifications to the cloned polypeptide including proper folding and glycosylation. For example, such mammalian host cells include COS-7 cells (e.g., ATCC CRL 1651), non-secreting myeloma cells (e.g., SP2/0-AG14; ATCC CRL 1581), Chinese hamster ovary cells (e.g., CHO-K1, ATCC CCL 61; CHO-DG44, Urlaub et al., Somat Cell Mol Genet. 12(6):555-66 (1986)), rat pituitary cells (e.g., GH_1; ATCC CCL 82), HeLa S3 cells (e.g., ATCC CCL 2.2), and rat hepatoma cells (e.g., H-4-II-E; ATCC CRL 1548).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, and simian virus. In addition, promoters from mammalian cells, such as actin, collagen, or myosin, can be employed. Alternatively, a prokaryotic promoter (such as the bacteriophage T3 RNA polymerase promoter) can be employed, wherein the prokaryotic promoter is

regulated by a eukaryotic promoter (for example, see Zhou et al., Mol. Cell. Biol. 10:4529-4537 (1990); Kaufman et al., Nucl. Acids Res. 19:4485-4490 (1991)). Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated.

In general, eukaryotic regulatory regions will include a promoter region sufficient to direct the initiation of RNA synthesis. Such a eukaryotic promoter can be, for example, the promoter of the mouse metallothionein I gene (Hamer et al., J. Mol. Appl. Gen. 1:273-288 (1982)); the TK promoter of Herpes virus (McKnight, Cell 31:355-365 (1982)); the SV40 early promoter (Benoist et al., Nature (London) 290:304-310 (1981)); the Rous sarcoma virus promoter ((Gorman, "High Efficiency Gene Transfer into Mammalian cells," in DNA Cloning: A Practical Approach, Volume II, Glover (Ed.), IRL Press, pp. 143-190 (1985)); the cytomegalovirus promoter (Foecking et al., Gene 45:101 (1980)); the yeast gal4 gene promoter (Johnston et al., Proc. Natl. Acad. Sci. USA 79:6971-6975 (1982); Silver et al., Proc. Natl. Acad. Sci. USA 81:5951-5955 (1984)); and the IgG promoter (Orlandi et al., Proc. Natl. Acad. Sci. USA 86:3833-3837 (1989)).

Strong regulatory sequences can be used. Examples of such regulatory sequences include the SV40 promoter-enhancer (Gorman, "High Efficiency Gene Transfer into Mammalian cells," in DNA Cloning: A Practical Approach, Volume II, Glover (Ed.), IRL Press, pp. 143-190 (1985)), the hCMV-MIE promoter-enhancer (Bebbington et al., Bio/Technology 10:169-175 (1992)), Chinese Hamster EF-1α promoter (see, e.g., U.S. Patent No. 5,888,809) and antibody heavy chain promoter (Orlandi et al., Proc. Natl. Acad. Sci. USA 86:3833-3837 (1989)). Also included are the kappa chain enhancer for the expression of the light chain and the IgH enhancer (Gillies, "Design of Expression Vectors and Mammalian Cell Systems Suitable for Engineered Antibodies," in Antibody Engineering: A Practical Guide, C. Borrebaeck (Ed.), W. H. Freeman and Company, pp. 139-157 (1992); Orlandi et al., supra).

The engineered antibody-encoding nucleic acid and an operably linked promoter may be introduced into eukaryotic cells as a non-replicating DNA molecule, which may either be a linear molecule or a circular molecule. Since such molecules are incapable of autonomous replication, the expression of the protein may occur through the transient expression of the introduced sequence. In one aspect, permanent expression occurs through the integration of the introduced sequence into the host chromosome.

In some embodiments, the introduced nucleic acid will be incorporated into a plasmid or viral vector that is capable of autonomous replication in the recipient host. Numerous possible vector systems are available for this purpose. One class of vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors relies upon the integration of the desired genomic or cDNA sequences into the host chromosome. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include those described by Okayama, Mol. Cell. Biol. 3:280 (1983), Sambrook et al., supra, Ausubel et al., supra, Bebbington et al., supra, Orlandi et al., supra, Fouser et al., Bio/Technology 10:1121-1127 (1992); and Gillies, supra. Genomic DNA expression vectors which include intron sequences are described by Orlandi et al., supra. Also, see generally, Lerner et al. (Eds.), New Techniques In Antibody Generation, Methods 2(2) (1991).

To obtain mammalian cells that express intact antibody, the expression vector comprising a nucleic acid encoding an antibody light chain can be co-transfected or transfected into mammalian cells with an antibody heavy chain expression vector. Alternatively, mammalian cells containing a heavy chain expression vector can be transfected with an antibody light chain expression vector, or mammalian cells containing an antibody light chain expression vector can be transfected with an antibody heavy chain expression vector. Moreover, mammalian cells can be transfected with a single expression vector comprising nucleic acid (e.g., DNA) fragments that encode an antibody light chain, as well as nucleic acid (e.g., DNA) fragments that encode antibody heavy chain. See, for example, Gillies, supra; Bebbington et al., supra. Any of these approaches will produce transfected cells that express whole engineered antibody molecules. Standard transfection and transformation techniques are well known in the art. See, for example, Sambrook et al., supra; Ausubel et al., supra.

An example of cell line development and protein expression is described in Example 1.

B. Methods for Isolating an Engineered Antibody from Transfected Cells

Transformed or transfected cells that carry the expression vector are selected using the appropriate drug. For example, G418 can be used to select transfected cells carrying

an expression vector having the aminoglycoside phosphotransferse gene. (See, e.g., Southern et al., J. Mol. Appl. Gen. 1:327-341 (1982).) Alternatively, hygromycin-B can be used to select transfected cells carrying an expression vector having the hygromycin-B-phosphotransferase gene. (See, e.g., Palmer et al., Proc. Natl. Acad. Sci. USA 84:1055-1059 (1987).) Aminopterin and mycophenolic acid can be used to select transfected cells carrying an expression vector having the xanthine-guanine phosphoribosyltransferase gene. (See, e.g., Mulligan et al., Proc. Natl. Acad. Sci. USA 78:2072-2076 (1981).) Methotrexate can be used to select transformed cells carrying an expression vector having the dihydrofolate reductase gene. (See, e.g., Wigler et al., Proc Natl. Acad. Sci. USA 77(6):3567-70 (1980).)

Transformed or transfected cells that produce the engineered antibody can be identified using a variety of methods. For example, any immunodetection assay can be used to identify such "transfectomas."

After transformants or transfectants have been identified, the cells are cultured and antibodies are isolated from the cells and/or the culture supernatants. Isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography. For example, see Coligan et al. (eds.), Current Protocols In Immunology, John Wiley and Sons (1991), for detailed protocols.

Methods for Preparing Immunoconjugates

A. Preparation of Antibody Fragments

The present invention also provides immunoconjugates of engineered antibodies or from antigen-binding antibody fragments. Antibody fragments can be obtained from, for example, recombinant host cells (e.g., transformants or transfectants) and/or by proteolytic cleavage of intact engineered antibodies. Antibody fragments can be obtained directly from transformants or transfectants by transfecting cells with a heavy chain structural gene that has been mutated. For example, transfectomas can produce Fab fragments if a stop codon is inserted following the sequence of the C_H1 domain. Alternatively, transfectomas can produce Fab' or $F(ab')_2$ fragments if a stop codon is inserted after the sequence encoding the hinge region of the heavy chain.

Alternatively, antibody fragments can be prepared from intact antibodies using well-known proteolytic techniques. For example, see, Coligan et al., supra. Moreover,

$F(ab')_2$ fragments can be obtained using pepsin digestion of intact antibodies. Divalent fragments can be cleaved to monovalent fragments using conventional disulfide bond reducing agents, e.g., dithiothreitol (DTT) and the like.

B. Methods of Conjugation

A wide variety of diagnostic, preventative and therapeutic agents can be advantageously conjugated to the antibodies of the invention. In some embodiments, can antibody can be stoichiometrically or fully-loaded (i.e., C# = Y, where Y refers to the average number of active moieties attached to each antibody molecule). In other embodiments, an antibody can be partially-loaded (i.e., C# > Y).

Immunoconjugates can be prepared by conjugating a diagnostic, preventative or therapeutic agent to an intact antibody, or antigen-binding fragment thereof. Such techniques are described in Shih et al., Int. J. Cancer 41:832-839 (1988); Shih et al., Int. J. Cancer 46:1101-1106 (1990); Shih et al., U.S. Pat. No. 5,057,313; Shih Cancer Res. 51:4192, International Publication WO 02/088172; U.S. Pat. No. 6,884,869; International Patent Publication WO 2005/081711; and U.S. Published Application 2003-0130189 A1, all of which are incorporated by reference herein.

In addition, those of skill in the art will recognize numerous possible variations of conjugation methods. For example, it is possible to construct a "divalent immunoconjugate" by attaching a diagnostic or therapeutic agent to a carbohydrate moiety and to a free sulfhydryl group.

In some embodiments, the interchain cysteine residues are present as a disulfide bond as a result of the oxidation of the thiol (--SH) side groups of the cysteine residues. Treatment of the disulfide bond with a reducing agent can causes reductive cleavage of the disulfide bonds to leave free thiol groups.

In some embodiments, the agent has, or is modified to include, a group reactive with an interchain cysteine residue. For example, an agent can be attached by conjugation to thiols. For examples of chemistries that can be used for conjugation, see, e.g., Current Protocols in Protein Science (John Wiley & Sons, Inc.), Chapter 15 (Chemical Modifications of Proteins) (the disclosure of which is incorporated by reference herein in its entirety).

For example, when chemical activation of the antibody results in formation of free thiol groups, the protein may be conjugated with a sulfhydryl reactive agent. In some embodiments, the agent is one which is substantially specific for free thiol groups. Such

agents include, for example, malemide, haloacetamides (e.g., iodo, bromo or chloro), haloesters (e.g., iodo, bromo or chloro), halomethyl ketones (e.g., iodo, bromo or chloro), benzylic halides (e.g., iodide, bromide or chloride), vinyl sulfone and pyridyithio.

In specific embodiments, the sulfyhydryl reactive agent can be an alpha-haloacetyl compounds such as iodoacetamide, maleimides such as N-ethylmaleimide, mercury derivatives such as 3,6-bis-(mercurimethyl)dioxane with counter ions of acetate, chloride or nitrate, and disulfide derivatives such as disulfide dioxide derivatives, polymethylene bismethane thiosulfonate reagents and crabescein (a fluorescent derivative of fluorescein containing two free sulfhydryl groups which have been shown to add across disulfide bonds of reduced antibody).

Alpha-haloacetyl compounds such as iodoacetate readily react with sulfhydryl groups to form amides. These compounds have been used to carboxymethylate free thiols. They are not strictly SH specific and will react with amines. The reaction involves nucleophilic attack of the thiolate ion resulting in a displacement of the halide. The reactive haloacetyl moiety, X--CH_2CO--, has been incorporated into compounds for various purposes. For example, bromotrifluoroacetone has been used for F-19 incorporation, and N-chloroacetyliodotyramine has been employed for the introduction of radioactive iodine into proteins.

Maleimides such as N-ethylmaleimide are considered to be fairly specific to sulfhydryl groups, especially at pH values below 7, where other groups are protonated. Thiols undergo Michael reactions with maleimides to yield exclusively the adduct to the double bond. The resulting thioether bond is very stable. They also react at a much slower rate with amino and imidazoyl groups. At pH 7, for example, the reaction with simple thiols is about 1,000 fold faster than with the corresponding amines. The characteristic absorbance change in the 300 nm region associated with the reaction provides a convenient method for monitoring the reaction. These compounds are stable at low pH but are susceptible to hydrolysis at high pH. See generally Wong, Chemistry of Protein Conjugation and Cross-linking; CRC Press, Inc., Boca Raton, 1991: Chapters 2 and 4.

An agent (such as a drug) which is not inherently reactive with sulfhydryls may still be conjugated to the chemically activated antibody by means of a bifunctional crosslinking agent which bears both a group reactive with the agent and a sulfhydryl reactive group. The cross-linking agent may be reacted simultaneously with both the molecule of interest (e.g., through an amino, carboxy or hydroxy group) and the

chemically activated protein, or it may be used to derivatize the molecule of interest to form a partner molecule which is then sulfhydryl reactive by virtue of a moiety derived from the agent, or it may be used to derivatize the chemically activated protein to make it reactive with the molecule of interest.

The agent also can be linked to an antibody by a linker. Suitable linkers include, for example, cleavable and non-cleavable linkers. A cleavable linker is typically susceptible to cleavage under intracellular conditions. Suitable cleavable linkers include, for example, a peptide linker cleavable by an intracellular protease, such as lysosomal protease or an endosomal protease. In exemplary embodiments, the linker can be a dipeptide linker, such as a valine-citrulline (val-cit) or a phenylalanine-lysine (phe-lys) linker. Other suitable linkers include linkers hydrolyzable at a pH of less than 5.5, such as a hydrazone linker. Additional suitable cleavable linkers include disulfide linkers.

A linker can include a group for linkage to the antibody. For example, a linker can include a sulfhydryl reactive group(s) (e.g., malemide, haloacetamides (e.g., iodo, bromo or chloro), haloesters (e.g., iodo, bromo or chloro), halomethyl ketones (e.g., iodo, bromo or chloro), benzylic halides (e.g., iodide, bromide or chloride), vinyl sulfone and pyridyithio). See generally Wong, Chemistry of Protein Conjugation and Cross-linking; CRC Press, Inc., Boca Raton, 1991.

In certain embodiments, the immunoconjugate has the following formula:

$$Ab_z\left(A_a-W_w-Y_y-D\right)_p$$

or pharmaceutically acceptable salts or solvates thereof,

wherein:

Ab is an antibody,

A is a stretcher unit,

a is 0 or 1,

each W is independently a linker unit,

w is an integer ranging from 0 to 12,

Y is a spacer unit, and

y is 0, 1 or 2,

p ranges from 1 to about 20, and

D is a diagnostic, preventative and therapeutic agent,

z is the number of predetermined conjugation sites on the protein.

When the antibody is fully loaded, p=z. When the antibody is partially loaded, p<z. In some embodiments, p is an even integer. In specific embodiments, p = 2, 4, 6 or 8. In a specific embodiment, p = z = 4. In other embodiments, 0 < p <8.

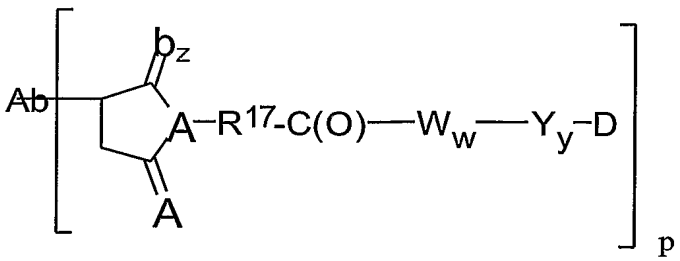
A stretcher unit can is capable of linking a linker unit to an antibody. The stretcher unit has a functional group that can form a bond with an interchain cysteine residue of the antibody. Useful functional groups include, but are not limited to, sulfhydryl reactive groups, as described above..

The linker unit is typically an amino acid unit, such as for example a dipeptide, tripeptide, tetrapeptide, pentapeptide, hexapeptide, heptapeptide, octapeptide, nonapeptide, decapeptide, undecapeptide or dodecapeptide unit. The linker unit can be cleavage or non-cleavable inside the cell.

In one embodiment, the amino acid unit is valine-citrulline. In another embodiment, the amino acid unit is phenylalanine-lysine. In yet another embodiment, the amino acid unit is N-methylvaline-citrulline. In yet another embodiment, the amino acid unit is 5-aminovaleric acid, homo phenylalanine lysine, tetraisoquinolinecarboxylate lysine, cyclohexylalanine lysine, isonepecotic acid lysine, beta-alanine lysine, glycine serine valine glutamine and isonepecotic acid. In certain embodiments, the Amino Acid unit can comprise natural amino acids. In other embodiments, the Amino Acid unit can comprise non-natural amino acids.

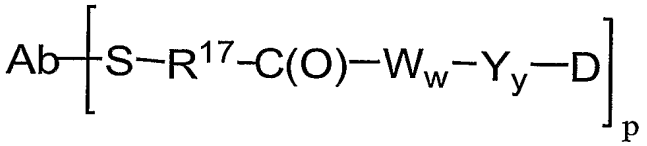
A spacer unit, if present, links a linker unit to D. Alternately, a spacer unit can link a stretcher unit to a drug moiety when the linker unit is absent. The spacer unit can also link a diagnostic, preventative and therapeutic agents to an antibody when both the linker unit and stretcher unit are absent. In one embodiment, the spacer unit is a p-aminobenzyl alcohol (PAB) unit, a p-aminobenzyl ether unit, or p-aminobenzyl carbamoyl unit. (See, e.g., U.S. Patent Publication Nos. 2003-0130189).

In some embodiments, the immunoconjugate has the formula:



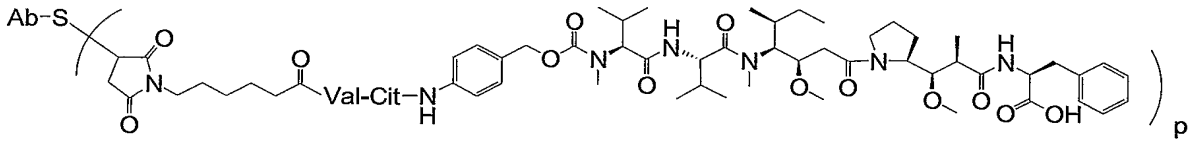
wherein R^{17} is selected from -C_1-C_{10} alkylene-, -C_3-C_8 carbocyclo-, -O-(C_1-C_8 alkyl)-, -arylene-, -C_1-C_{10} alkylene-arylene-, -arylene-C_1-C_{10} alkylene-, -C_1-C_{10} alkylene-(C_3-C_8 carbocyclo)-, -(C_3-C_8 carbocyclo)-C_1-C_{10} alkylene-, -C_3-C_8 heterocyclo-, -C_1-C_{10} alkylene-(C_3-C_8 heterocyclo)-, -(C_3-C_8 heterocyclo)-C_1-C_{10} alkylene-, -$(CH_2CH_2O)_r$-, and -$(CH_2CH_2O)_r$-CH_2-. In some embodiments, R^{17} is -$(CH_2)_5$- or $(CH_2CH_2O)_r$-CH_2- and r is 2.

In another embodiment, the immunoconjugate has the formula:

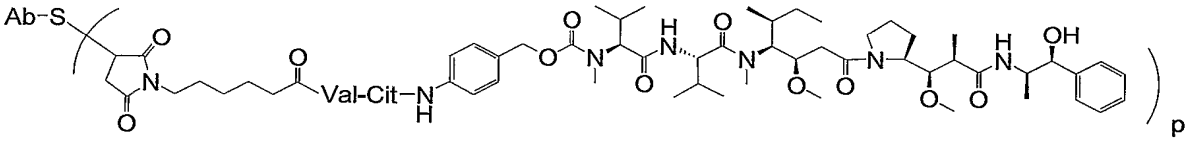


wherein R^{17} is as defined above.

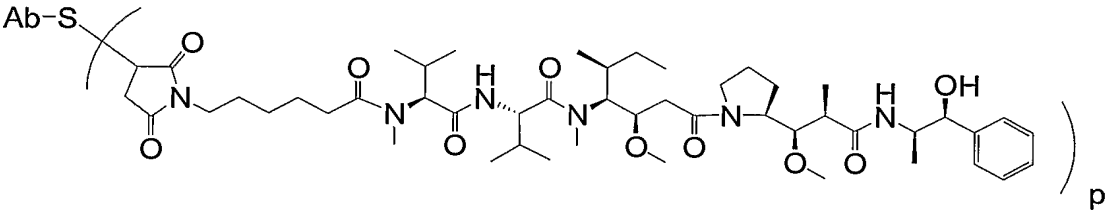
In additional embodiments, the immunoconjugate has one of the following formulae:



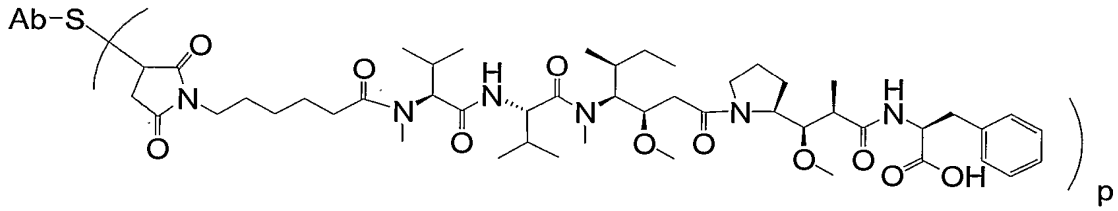
Ab-MC-vc-PAB-MMAF



Ab-MC-vc-PAB-MMAE



Ab-MC-MMAE



Ab-MC-MMAF

The final immunoconjugate may be purified using conventional techniques, such as sizing chromatography on Sephacryl S-300, affinity chromatography such as protein A or protein G sepharose, or the like.

Examples of protein purification and conjugation is described in Examples 1 and 2.

Use of Immunoconjugates for Diagnosis and Therapy

A. Use of Immunoconjugates for Diagnosis

The immunoconjugates can be used for diagnostic imaging. For example, the immunoconjugate can be a radiolabeled monoclonal antibody. See, for example, Srivastava (ed.), Radiolabeled Monoclonal Antibodies For Imaging And Therapy, Plenum Press (1988); Chase, "Medical Applications of Radioisotopes," in Remington's Pharmaceutical Sciences, 18th Edition, Gennaro et al. (eds.), Mack Publishing Co., pp. 624-652 (1990); and Brown, "Clinical Use of Monoclonal Antibodies," in Biotechnology and Pharmacy, Pezzuto et al. (eds.), Chapman and Hall, pp. 227-249 (1993). This technique, also known as immunoscintigraphy, uses a gamma camera to detect the location of gamma-emitting radioisotopes conjugated to monoclonal antibodies. Diagnostic imaging can be used to diagnose cancer, autoimmune disease, infectious disease and/or cardiovascular disease. (See, e.g., Brown, supra.)

In one example, the immunoconjugates can be used to diagnose cardiovascular disease. For example, immunoconjugates comprising anti-myosin antibody fragments can be used for imaging myocardial necrosis associated with acute myocardial infarction. Immunoconjugates comprising antibody fragments that bind to platelets or fibrin can be used for imaging deep-vein thrombosis. Moreover, immunoconjugates comprising antibody fragments that bind to activated platelets can be used for imaging atherosclerotic plaque.

Immunoconjugates can also be used in the diagnosis of infectious diseases. For example, immunoconjugates comprising antibody fragments that bind specific bacterial antigens can be used to localize abscesses. In addition, immunoconjugates comprising

antibody fragments that bind granulocytes and inflammatory leukocytes can be used to localize sites of bacterial infection.

Numerous studies have evaluated the use of monoclonal antibodies for scintigraphic detection of cancer. See, for example, Brown, supra. Investigations have covered the major types of solid tumors such as melanoma, colorectal carcinoma, ovarian carcinoma, breast carcinoma, sarcoma, and lung carcinoma. Thus, the present invention also contemplates the detection of cancer using immunoconjugates comprising antibody fragments that bind tumor markers to detect cancer. Examples of such tumor markers include carcinoembryonic antigen, alpha-fetoprotein, oncogene products, tumor-associated cell surface antigens, and necrosis-associated intracellular antigens, as well as the tumor-associated antigens and tumor-specific antigens discussed infra.

In addition to diagnosis, monoclonal antibody imaging can be used to monitor therapeutic responses, detect recurrences of a disease, and guide subsequent clinical decisions.

For diagnostic and monitoring purposes, radioisotopes may be bound to antibody fragments either directly or indirectly by using an intermediary functional group. Such intermediary functional groups include, for example, DTPA (diethylenetriaminepentaacetic acid) and EDTA (ethylene diamine tetraacetic acid). The radiation dose delivered to the patient is typically maintained at as low a level as possible. This may be accomplished through the choice of isotope for the best combination of minimum half-life, minimum retention in the body, and minimum quantity of isotope which will permit detection and accurate measurement. Examples of radioisotopes which can be bound to antibodies and are appropriate for diagnostic imaging include ^{99}mTc and ^{111}In.

Studies indicate that antibody fragments, particularly Fab and Fab', provide suitable tumor/background ratios. (See, e.g., Brown, supra.)

The immunoconjugates also can be labeled with paramagnetic ions for purposes of in vivo diagnosis. Elements which are particularly useful for Magnetic Resonance Imaging include Gd, Mn, Dy, and Fe ions.

The immunoconjugates can also detect the presence of particular antigens in vitro. In such immunoassays, the immunoconjugates may be utilized in liquid phase or bound to a solid-phase carrier. For example, an intact antibody, or antigen-binding fragment thereof, can be attached to a polymer, such as aminodextran, in order to link the antibody component to an insoluble support such as a polymer-coated bead, plate, or tube.

Alternatively, the immunoconjugates can be used to detect the presence of particular antigens in tissue sections prepared from a histological specimen. Such in situ detection can be accomplished, for example, by applying a detectably-labeled immunoconjugate to the tissue sections. In situ detection can be used to determine the presence of a particular antigen and to determine the distribution of the antigen in the examined tissue. General techniques of in situ detection are well known to those of ordinary skill. (See, e.g., Ponder, "Cell Marking Techniques and Their Application," in Mammalian Development: A Practical Approach, Monk (ed.), IRL Press, pp. 115-138 (1987); Coligan et al., supra.)

Detectable labels such as enzymes, fluorescent compounds, electron transfer agents, and the like can be linked to a carrier by conventional methods well known to the art. These labeled carriers and the immunoconjugates prepared from them can be used for in vitro immunoassays and for in situ detection, much as an antibody conjugate can be prepared by direct attachment of the labels to antibody. The loading of the immunoconjugate with a plurality of labels can increase the sensitivity of immunoassays or histological procedures, where only a low extent of binding of the antibody, or antibody fragment, to target antigen is achieved.

B. Use of Immunoconjugates for Therapy

Immunoconjugates can be used to treat viral and bacterial infectious diseases, cardiovascular disease, autoimmune disease, and cancer. The objective of such therapy is to deliver cytotoxic or cytostatic doses of an active agent (e.g., radioactivity, a toxin, or a drug) to target cells, while minimizing exposure to non-target tissues.

A radioisotope can be attached to an intact antibody, or antigen-binding fragment thereof, directly or indirectly, via a chelating agent. For example, ^{67}Cu can be conjugated to an antibody component using the chelating agent, p-bromo-acetamidobenzyl-tetraethylaminetetraacetic acid (TETA). (See, e.g., Chase, supra.)

Moreover, immunoconjugates can be prepared in which the therapeutic agent is a toxin or drug. Useful toxins for the preparation of such immunoconjugates include ricin, abrin, pokeweed antiviral protein, gelonin, diphtherin toxin, and Pseudomonas endotoxin. Useful chemotherapeutic drugs for the preparation of immunoconjugates include auristatin, dolastatin, MMAE, MMAF, AFP, AEB, doxorubicin, daunorubicin, methotrexate, melphalin, chlorambucil, vinca alkaloids, 5-fluorouridine, mitomycin-C, taxol, L-asparaginase, mercaptopurine, thioguanine, hydroxyurea, cytarabine,

cyclophosphamide, ifosfamide, nitrosoureas, cisplatin, carboplatin, mitomycin, dacarbazine, procarbizine, topotecan, nitrogen mustards, cytoxan, etoposide, BCNU, irinotecan, camptothecins, bleomycin, idarubicin, dactinomycin, plicamycin, mitoxantrone, asparaginase, vinblastine, vincristine, vinorelbine, paclitaxel, and docetaxel and salts, solvents and derivatives thereof. Other suitable agents include chelators, such as DTPA, to which detectable labels such as fluorescent molecules or cytotoxic agents such as heavy metals or radionuclides can be complexed; and toxins such as Pseudomonas exotoxin, and the like.

In some embodiments, the diagnostic, preventative or therapeutic agent is auristatin E (also known in the art as dolastatin-10) or a derivative thereof as well as pharmaceutically salts or solvates thereof. Typically, the auristatin E derivative is, e.g., an ester formed between auristatin E and a keto acid. For example, auristatin E can be reacted with paraacetyl benzoic acid or benzoylvaleric acid to produce AEB and AEVB, respectively. Other typical auristatin derivatives include AFP, MMAF, and MMAE. The synthesis and structure of auristatin E and its derivatives, as well as linkers, are described in U.S. Patent Application No. 09/845,786 (U.S. Patent Application Publication No. 20030083263), U.S. Patent Application Publication No. 2005-0238629; International Patent Application No. PCT/US03/24209; International Patent Application No. PCT/US02/13435; International Patent Application No. PCT/US02/13435; International Patent Publication No. WO 04/073656; and U.S. Patent Nos. 6,884,869; 6,323,315; 6,239,104; 6,214,345; 6,034,065; 5,780,588; 5,665,860; 5,663,149; 5,635,483; 5,599,902; 5,554,725; 5,530,097; 5,521,284; 5,504,191; 5,410,024; 5,138,036; 5,076,973; 4,986,988; 4,978,744; 4,879,278; 4,816,444; and 4,486,414 (all of which are incorporated by reference herein in their entirety).

In some embodiments, the anti-cancer agent includes, but is not limited to, a drug listed in Drug Table below.

Drug Table

Alkylating agents	
Nitrogen mustards:	cyclophosphamide
	Ifosfamide
	trofosfamide
	Chlorambucil
Nitrosoureas:	carmustine (BCNU)
	Lomustine (CCNU)
Alkylsulphonates	busulfan

	Treosulfan
Triazenes:	Dacarbazine
Platinum containing compounds:	Cisplatin
	carboplatin
Plant Alkaloids	
Vinca alkaloids:	vincristine
	Vinblastine
	Vindesine
	Vinorelbine
Taxoids:	paclitaxel
	Docetaxol
DNA Topoisomerase Inhibitors	
Epipodophyllins:	etoposide
	Teniposide
	Topotecan
	9-aminocamptothecin
	camptothecin
	crisnatol
mitomycins:	Mitomycin C
Anti-metabolites	
Anti-folates:	
DHFR inhibitors:	methotrexate
	Trimetrexate
IMP dehydrogenase Inhibitors:	mycophenolic acid
	Tiazofurin
	Ribavirin
	EICAR
Ribonuclotide reductase Inhibitors:	hydroxyurea
	deferoxamine
Pyrimidine analogs:	
Uracil analogs	5-Fluorouracil
	Floxuridine
	Doxifluridine
	Ratitrexed
Cytosine analogs	cytarabine (ara C)
	Cytosine arabinoside
	fludarabine
Purine analogs:	mercaptopurine
	Thioguanine
Hormonal therapies:	
Receptor antagonists:	
Anti-estrogen	Tamoxifen
	Raloxifene
	megestrol
LHRH agonists:	goscrclin
	Leuprolide acetate
Anti-androgens:	flutamide

	bicalutamide
Retinoids/Deltoids	
Vitamin D3 analogs:	EB 1089
	CB 1093
	KH 1060
Photodynamic therapies:	vertoporfin (BPD-MA)
	Phthalocyanine
	photosensitizer Pc4
	Demethoxy-hypocrellin A
	(2BA-2-DMHA)
Cytokines:	Interferon- α
	Interferon- γ
	Tumor necrosis factor
Others:	
Isoprenylation inhibitors:	Lovastatin
Dopaminergic neurotoxins:	1-methyl-4-phenylpyridinium ion
Cell cycle inhibitors:	staurosporine
Actinomycins:	Actinomycin D
	Dactinomycin
Bleomycins:	bleomycin A2
	Bleomycin B2
	Peplomycin
Anthracyclines:	daunorubicin
	Doxorubicin (adriamycin)
	Idarubicin
	Epirubicin
	Pirarubicin
	Zorubicin
	Mitoxantrone
MDR inhibitors:	verapamil
Ca^{2+}ATPase inhibitors:	thapsigargin

In some embodiments, the diagnostic, preventative or therapeutic agent is not a radioisotope.

In some embodiments, an immunoconjugate can be used to treat one of the following particular types of cancer:

Solid tumors, including but not limited to:

sarcoma

fibrosarcoma

myxosarcoma

liposarcoma

chondrosarcoma

osteogenic sarcoma

chordoma
angiosarcoma
endotheliosarcoma
lymphangiosarcoma
lymphangioendotheliosarcoma
synovioma
mesothelioma
Ewing's tumor
leiomyosarcoma
rhabdomyosarcoma
colon cancer
colorectal cancer
kidney cancer
pancreatic cancer
bone cancer
breast cancer
ovarian cancer
prostate cancer
esophogeal cancer
stomach cancer (e.g., gastrointestinal cancer)
oral cancer
nasal cancer
throat cancer
squamous cell carcinoma (e.g., of the lung)
basal cell carcinoma
adenocarcinoma (e.g., of the lung)
sweat gland carcinoma
sebaceous gland carcinoma
papillary carcinoma
papillary adenocarcinomas
cystadenocarcinoma
medullary carcinoma
bronchogenic carcinoma
renal cell carcinoma

hepatoma
bile duct carcinoma
choriocarcinoma
seminoma
embryonal carcinoma
Wilms' tumor
cervical cancer
uterine cancer
testicular cancer
small cell lung carcinoma
bladder carcinoma
lung cancer
non-small cell lung cancer
epithelial carcinoma
glioma
glioblastoma multiforme
astrocytoma
medulloblastoma
craniopharyngioma
ependymoma
pinealoma
hemangioblastoma
acoustic neuroma
oligodendroglioma
meningioma
skin cancer
melanoma
neuroblastoma
retinoblastoma
blood-borne cancers, including but not limited to:
acute lymphoblastic leukemia "ALL"
acute lymphoblastic B-cell leukemia
acute lymphoblastic T-cell leukemia
acute myeloblastic leukemia "AML"

acute promyelocytic leukemia "APL"
acute monoblastic leukemia
acute erythroleukemic leukemia
acute megakaryoblastic leukemia
acute myelomonocytic leukemia
acute nonlymphocyctic leukemia
acute undifferentiated leukemia
chronic myelocytic leukemia "CML"
chronic lymphocytic leukemia "CLL"
hairy cell leukemia
multiple myeloma
acute and chronic leukemias:
lymphoblastic
myelogenous
lymphocytic
myelocytic leukemias
Lymphomas:
Hodgkin's disease
non-Hodgkin's Lymphoma
Multiple myeloma
Waldenström's macroglobulinemia
Heavy chain disease
Polycythemia vera
Other cancers:
Peritoneal cancer
Hepatocellular cancer
Hepatoma
Salivary cancer
Vulval cancer
Thyroid
Penile cancer
Anal cancer
Head and neck cancer
Renal cell carcinoma

Acute anaplastic large cell carcinoma

Cutaneous anaplastic large cell carcinoma

In some embodiments, an immunoconjugate can be used to treat one of the following particular types of autoimmune disease:

Active Chronic Hepatitis

Addison's Disease

Allergic Alveolitis

Allergic Reaction

Allergic Rhinitis

Alport's Syndrome

Anaphlaxis

Ankylosing Spondylitis

Anti-phosholipid Syndrome

Arthritis

Ascariasis

Aspergillosis

Atopic Allergy

Atropic Dermatitis

Atropic Rhinitis

Behcet's Disease

Bird-Fancier's Lung

Bronchial Asthma

Caplan's Syndrome

Cardiomyopathy

Celiac Disease

Chagas' Disease

Chronic Glomerulonephritis

Cogan's Syndrome

Cold Agglutinin Disease

Congenital Rubella Infection

CREST Syndrome

Crohn's Disease

Cryoglobulinemia

Cushing's Syndrome
Dermatomyositis
Discoid Lupus
Dressler's Syndrome
Eaton-Lambert Syndrome
Echovirus Infection
Encephalomyelitis
Endocrine opthalmopathy
Epstein-Barr Virus Infection
Equine Heaves
Erythematosis
Evan's Syndrome
Felty's Syndrome
Fibromyalgia
Fuch's Cyclitis
Gastric Atrophy
Gastrointestinal Allergy
Giant Cell Arteritis
Glomerulonephritis
Goodpasture's Syndrome
Graft v. Host Disease
Graves' Disease
Guillain-Barre Disease
Hashimoto's Thyroiditis
Hemolytic Anemia
Henoch-Schonlein Purpura
Idiopathic Adrenal Atrophy
Idiopathic Pulmonary Fibritis
IgA Nephropathy
Inflammatory Bowel Diseases
Insulin-dependent Diabetes Mellitus
Juvenile Arthritis
Juvenile Diabetes Mellitus (Type I)
Lambert-Eaton Syndrome

Laminitis
Lichen Planus
Lupoid Hepatitis
Lupus
Lymphopenia
Meniere's Disease
Mixed Connective Tissue Disease
Multiple Sclerosis
Myasthenia Gravis
Pernicious Anemia
Polyglandular Syndromes
Presenile Dementia
Primary Agammaglobulinemia
Primary Biliary Cirrhosis
Psoriasis
Psoriatic Arthritis
Raynauds Phenomenon
Recurrent Abortion
Reiter's Syndrome
Rheumatic Fever
Rheumatoid Arthritis
Sampter's Syndrome
Schistosomiasis
Schmidt's Syndrome
Scleroderma
Shulman's Syndrome
Sjorgen's Syndrome
Stiff-Man Syndrome
Sympathetic Ophthalmia
Systemic Lupus Erythematosis
Takayasu's Arteritis
Temporal Arteritis
Thyroiditis
Thrombocytopenia

Thyrotoxicosis
Toxic Epidermal Necrolysis
Type B Insulin Resistance
Type I Diabetes Mellitus
Ulcerative Colitis
Uveitis
Vitiligo
Waldenstrom's Macroglobulemia
Wegener's Granulomatosis

The use of the immunoconjugates for the treatment of other cancers or autoimmune disorders is also contemplated and within the scope of the present invention.

C. Administration of Immunoconjugates

Generally, the dosage of administered immunoconjugate will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition, and previous medical history. Typically, it is desirable to provide the recipient with a dosage of immunoconjugate which is in the range of from about 1 pg/kg to 20 mg/kg (amount of agent/body weight of patient), although a lower or higher dosage may also be administered. For example, many studies have demonstrated successful diagnostic imaging with doses of 0.1 to 1.0 milligram, while other studies have shown improved localization with doses in excess of 10 milligrams. (See, e.g., Brown, supra.)

For therapeutic applications, generally about 10-200 milligrams of immunoconjugate will be administered, depending on protocol. In some embodiments, a dose is from about 0.5 mg/kg to about 20 mg/kg, or about 1 mg/kg to about 10 mg/kg or about 15 mg/kg. Some protocols provide for the administration daily for a period of several days, several weeks or several months. In some embodiments, an immunoconjugate is administered daily, 1-3 times per week, weekly, biweekly or monthly. To reduce patient sensitivity, it may be necessary to reduce the dosage and/or use antibodies from other species and/or use hypoallergenic antibodies, e.g., hybrid human or primate antibodies.

Administration of immunoconjugates to a patient can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional catheter, or by direct intralesional injection. When administering

immunoconjugates by injection, the administration may be by continuous infusion, or by single or multiple boluses.

The immunoconjugates can be formulated according to known methods to prepare pharmaceutically useful compositions, such as a medicament, whereby immunoconjugates are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline is one example of a pharmaceutically acceptable carrier. Other suitable carriers are well-known to those in the art. (See, e.g., Remington's Pharmaceutical Sciences, 18th Ed. (1990).)

For purposes of immunotherapy, an immunoconjugate and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A "therapeutically effective amount" is the amount administered that is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

Additional pharmaceutical methods may be employed to control the duration of action of an immunoconjugate in a therapeutic application. Control release preparations can be prepared through the use of polymers to complex or adsorb an immunoconjugate. For example, biocompatible polymers include matrices of poly(ethylene-co-vinyl acetate) and matrices of a polyanhydride copolymer of a stearic acid dimer and sebacic acid. (See, e.g., Sherwood et al., Bio/Technology 10:1446-1449 (1992).) The rate of release of an immunoconjugate from such a matrix depends upon the molecular weight of the immunoconjugate, the amount of immunoconjugate within the matrix, and the size of dispersed particles. (See, e.g., Saltzman et al., Biophysical. J. 55:163-171 (1989); and Sherwood et al., supra.) Other solid dosage forms are described in Remington's Pharmaceutical Sciences, 18th Ed. (1990).

The present invention is not to be limited in scope by the specific embodiments described herein. Various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

The invention is further described in the following examples, which are not intended to limit the scope of the invention. Cell lines described in the following examples were maintained in culture according to the conditions specified by the American Type Culture Collection (ATCC) or Deutsche Sammlung von Mikroorganismen

und Zellkulturen GmbH, Braunschweig, Germany (DMSZ). Cell culture reagents were obtained from Invitrogen Corp., Carlsbad, CA.

EXAMPLES

Example 1.

Construction and Expression of cAC10 Cysteine Variants

Procedures

Construction of chimeric AC10 (cAC10) from the AC10 hybridoma and expression of cAC10 in a CHO cell line has been described (Wahl et al., Cancer Res. 62(13): 3736-42 (2002)).

(i) Mutagenesis and Cloning

Mutants of cAC10 were generated in pBluescript vectors containing cDNAs for either cAC10 heavy (SEQ ID NO:6) (in pBSSK-AC10H) or cAC10 light (SEQ ID NO:8) (in pBSSK-AC10L) chain and encoding the cAC10 heavy (SEQ ID NO:7) or cAC10 light (SEQ ID NO:9) chain, respectively . Mutagenesis was performed using the Quikchange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. A pBluescript vector containing the cAC10 heavy chain cDNA, pBSSK AC10H shown in Figure 4, was used as a template to generate heavy chain C226S, C229S double mutant (having cysteine to serine substitutions are positions 226 and 229). (Residue numbering is of the mature cAC10 heavy and light chains, excluding the signal sequences.) Primer C226S:C229S: 5'GACAAAACTCACACA**TCC**CCACCG**TCC**CCAGCACCTGAACTC (SEQ ID NO:1) and its reverse complement partner were used to introduce the amino acid substitutions (the mutated codons are underlined). The resulting plasmid was called pBSSK AC10H226,229, containing the cDNA for cAC10 H226/229 (SEQ ID NO: 14) and encoding the cAC10 heavy chain C226S, C229S double mutant (SEQ ID NO:15).

A cAC10 heavy chain C220S mutant was generated using pBSSK AC10H as a template and primer C220S: 5'GTTGAGCCCAAATCT**TCT**GACAAAACTCA-CACATGCCC (SEQ ID NO:2) and its reverse complement partner (the mutated codon is underlined) to produce construct pBSSK AC10H220 containing the cDNA for cAC10 H220 (SEQ ID NO:10) and encoding the cAC10 C220S mutant (SEQ ID NO:11). pBSSK AC10H220 was used as a template to generate heavy chain C220S, C226S double mutant

using primer C226S: 5'GACAAAACTCACACA**TCC**CCACCG-TGCCCAGC (SEQ ID NO:3) and its reverse complement partner (the second mutated codon is underlined). The resulting plasmid was called pBSSK AC10H220,226, containing the cDNA for cAC10 H220/226 (SEQ ID NO:12) and encoding the cAC10 heavy chain C220S, C226S double mutant (SEQ ID NO:13). pBSSK AC10 H226,229 was used as a template to generate heavy chain C220S, C226S, C229S mutant using primer C220S: 5'GTTGAGCCCAAATCT**TCT**GACAAAACTCACACATCCCC (SEQ ID NO:4) and its reverse complement partner (the mutated codon is underlined). The resulting plasmid was called pBSSK AC10 H220,226,229, containing the cDNA for cAC10 H220/226/229 (SEQ ID NO:16) and encoding the cAC10 heavy chain C220S, C226S, C229S mutant (SEQ ID NO:17).

A pBluescript vector containing the cAC10 light chain cDNA, pBSSK AC10L as shown in Figure 5, was used as a template to generate light chain C218S mutant pBSSK AC10L218 using primer C218S: 5'CTTCAACAGGGGAGAG**TCT**TAGACGCG-TATTGG (SEQ ID NO:5) and its reverse complement partner (the mutated codon is underlined). The resulting plasmid was called pBSSK AC10L218, containing the cDNA for cAC10 L218 (SEQ ID NO:18) and encoding the cAC10 light chain C218S mutant (SEQ ID NO:19).

cAC10 heavy chain parent and cysteine variant cDNAs were released from pBluescript by cleavage with restriction enzymes *XhoI* and *XbaI* and ligated into the pDEF38 expression vector (Running Deer and Allison, Biotechnol Prog. 20(3):880-9 (2004)) downstream of the CHEF EF-1α promoter. cAC10 light chain parent and cysteine variant cDNAs were released from pBluescript with *MluI* and cloned into the *MluI* site of pDEF38 downstream of the CHEF EF-1α promoter.

(ii) Stable cell line development and protein expression

The cAC10 variants were stably expressed in a CHO-DG44 cell line as previously described for the cAC10 parent antibody (Wahl et al., Cancer Res. 62:3736-3742 (2002)). pDEF38 expression constructs were linearized with restriction enzyme PvuI prior to transfection. Fifty micrograms of linearized pDEF38 cAC10 H chain parent or the cysteine variant construct was cotransfected with 50 μg of linearized pDEF38 cAC10 L chain parent or the cysteine variant construct into CHO-DG44 cells (Urlaub et al., Somat

Cell Mol Genet. 12(6):555-66 (1986)) by electroporation. Following electroporation, the cells were allowed to recover for 2 days in EX-CELL 325 PF CHO medium containing hypoxanthine and thymidine (JRH Bioscience, Lenexa, KS) and 4 mM L-glutamine (Invitrogen, Carlsbad, CA). After 2 days, stable cell lines expressing the cAC10 variants were selected by replacing the medium with selective medium without hypoxanthine and thymidine. Only cells that incorporated the plasmid DNA, which includes the selectable marker, were able to grow in the absence of hypoxanthine and thymidine. After cells were recovered, stable pools were scaled up to 30 ml shake flask cultures. Cell cloning was performed using a limited dilution method in a background of non-transfected CHO-DG44 feeder cells. Briefly, 0.5 transfected cells and 1000 non-transfected cells were plated per well of a microtiter plate in EX-CELL 325 PF CHO medium in the absence of hypoxanthine and thymidine. Following 7-10 days incubation individual colonies were picked and expanded. High titer clones were selected and cultured in spinners at a final volume of 2.5 L or WAVE bioreactors (WAVE Biotech LLC, Bridgewater, NJ) at a final volume of 5-10 L.

Results

cAC10, is a chimeric IgG_1 that binds to human CD30 (Wahl et al., supra). Antibody cAC10, has 4 solvent accessible inter-chain disulfide bonds that are readily reducible and conjugated to vcMMAE, a thiol-reactive auristatin drug in near quantitative yield (Doronina et al., Nat. Biotechnol. 21:778-784 (2003)). This ADC comprising the cAC10 parent antibody with all 8 accessible cysteines and loaded with vcMMAE is designated here as C8-E8 (Figure 1A). The accessible cysteines in cAC10 were systematically mutated to a homologous residue, serine, to generate antibody variants with either 4 (C4v1, C4v2 and C4v3) or 2 (C2v1 and C2v2) remaining accessible cysteines (Table 1 and Figure 1A). In addition, antibody variant C6v1 with heavy chain cysteine residue 226 changed to serine (not shown) had six accessible cysteines. These engineered antibody variants provided a starting point to create conjugates with precisely defined stoichiometry and site of drug attachment.

All antibody variants were expressed in stable CHO-DG44 cell lines at titers of 25-125 mg/L. The antibody variants were purified from 2.5 to 10 L cultures by protein A affinity and ion exchange chromatography (Table 1) and then analyzed by size exclusion chromatography and SDS-PAGE. All antibody variants, except C4v3, were estimated to be > 98 % monomeric by size exclusion chromatography (Table 2). All variants

electrophoresed under reducing conditions gave rise to two major bands consistent with the presence of heavy and light chains (data not shown). As for SDS-PAGE under non-reducing conditions, all antibody variants (except C4v3) gave electrophoretic patterns (Figure 1B) consistent with the anticipated inter-chain disulfide bonding pattern (Figure 1A). Antibody variant C4v3 was excluded from the remainder of these studies on the basis of its unanticipated electrophoretic behavior and a size exclusion chromatography profile that suggested significant aggregation.

Table 1
Generation and Characterization of Antibody Variants

cAC10 variant*	Location of Cys→Ser mutations†	Competition binding to Karpas-299 (IC_{50}, nM) #
C8	none (parent)	2.8 ± 0.1
C2v1	L214, H220, H226	2.2 ± 0.4
C2v2	H220, H226, H229	2.6 ± 0.4
C4v1	L214, H220	3.2 ± 0.4
C4v2	H226, H229	2.4 ± 0.1
C4v3	H220, H226	nd

*cAC10 variants are identified by the number of solvent accessible cysteine residues and, where necessary, a variant number. E.g., C2v1 denotes a cAC10 variant containing 2 solvent accessible cysteine residues (Figure 1A).
†L, light chain; H, heavy chain; numbering scheme of Kabat et al. (Sequences of Proteins of Immunological Interest, 5th ed. NIH, Bethesda, MD (1991)).
Mean (± SEM) for ≥ 3 independent experiments.
‖nd, not determined due to presence of shoulder and broadening of peak

Table 2
Protein recovery for each cAC10 cysteine variant following Protein A purification and results from size exclusion chromatography analysis.

cAC10 cys variant	**Protein recovery in mg/L culture (Protein A purified)**	**% monomer**
C2v1	120	98.8%
C2v2	92	99.1%
C4v1	33	99.5%
C4v2	36	94.6%
C4v3	37	*
C6v1	28	97.3%

* not determined due to presence of shoulder and broadening of peak.

Purified proteins were analyzed by SDS-PAGE under reducing and non-reducing conditions. All variants except cAC10 C4v3 displayed the expected banding pattern under non-reducing conditions as shown in Table 3 and Figure 1B.

Table 3
Expected band patterns and molecular weights for variants analyzed under non-reducing conditions by SDS-PAGE

cAC10 Variant	Cysteine mutations	Non-reduced band pattern	MWs (kDa)
C2v1	L218, H220/226	HH + L	98 + 24
C2v2	H220/226/229	H + L	49 + 24
C4v1	L218, H220	HH + L	98 + 24
C4v2	H226/229	HL	73
C4v3	H220/226	HH + L	98 + 24
C6v1	H226	HHLL	146

Aggregation was assessed by size exclusion high-performance liquid chromatography and all variants except cAC10 C4v3 were determined to be > 94 % monomeric. cAC10 C4v3 was found to be heterogeneous by both non-reducing SDS-PAGE and size exclusion analysis. The banding pattern of cAC10 C4v3 under non-reducing conditions included the expected heavy-heavy chain dimer and light chain bands as shown in Table 3 but also a heavy-light chain dimer and heavy chain alone suggesting that the free light chain cysteine was capable of forming a disulfide bond with the heavy chain cysteine at position H229.

Preparation and analysis of antibody drug conjugates

Procedures

(i) Preparation of antibody drug conjugates

cAC10 parent and cysteine variant antibodies were purified using Protein A chromatography and analyzed by SDS-PAGE and size exclusion chromatography. All cAC10 cysteine variants except cAC10 C6v1 were reduced using 10 mM dithiothreitol (DTT; Sigma, St Louis, MO), which was an excess over antibody of approximately 100X, in 0.025 M sodium borate pH 8, 0.025 M NaCl, and 1 mM diethylenetriaminepentaacetic acid (DTPA; Aldrich, Milwaukee, WI) for 1 h at 37 °C. The reduced antibody was diluted to 150 mL with water and applied to a 70 mL hydroxyapatite column (Macroprep ceramic

type I 40 μm, BioRad) at a flow rate of 10 mL/min. The column was previously equilibrated with 5 column volumes of 0.5 M sodium phosphate pH 7, 10 mM NaCl and 5 column volumes of 10 mM sodium phosphate pH 7, 10 mM NaCl. Following application, the column was washed with 5 column volumes of 10 mM sodium phosphate pH 7, 10 mM NaCl and then eluted with 100 mM sodium phosphate pH 7, 10 mM NaCl. Reduced antibody was titrated with 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB; Pierce) to determine the concentration of antibody-cysteine thiols. Reduced antibody was cooled to 0 °C and treated with 2.75 equivalents of maleimidocaproyl-valine-citrulline-p-aminobenyzoyl-MMAE (vcMMAE) in DMSO. The final DMSO concentration was 10% to ensure that the drug was fully soluble. After 40 min at 0 °C, excess cysteine was added to quench any unreacted vcMMAE and the mixture was diluted to 250 mL with water. The conjugate was purified on a hydroxyapatite column as described above. Antibody-drug conjugates were concentrated and the buffer changed to PBS using 15 mL Amicon Ultrafree 30K cutoff spin concentration devices. cAC10 C6v1 was reduced with a limited number of equivalents of TCEP (tris(2-carboxyethyl)phosphine, Acros) and conjugated to vcMMAE without removal of excess TCEP as follows: 35 mL of C6v1 (2.1 mg/mL or 14.3 μM; 74 mg) were treated with 4.0 equivalents of TCEP (57.1 μM, from 100 mM stock) in PBS with 1 mM DTPA for 2.5 h at 37 °C.

The extent of reduction was checked by purifying a small amount of the reduction reaction through a PD-10 column (Amersham Biosciences) and titrating the number of antibody-cysteine thiols with DTNB, yielding 5.7 per C6v1 The reduced antibody was then cooled to 0 °C and treated with 8.0 equivalents of vcMMAE (the concentration of antibody thiols was 73.5 μM and the vcMMAE concentration was 103.2 μM) in 3.9 mL of DMSO. After 135 min at 0 °C, 0.4 mL of 100 mM cysteine was added to quench any unreacted vcMMAE and the mixture was diluted to 250 mL with water. The conjugate was purified on a hydroxyapatite column as described above. cAC10-C6v1-vcMMAE (20 mL of 2.4 mg/mL; 48 mg) (C6v1-E6) was concentrated and the buffer changed to PBS using 15 mL Amicon Ultrafree 30K cutoff spin concentration devices.

The generation of parent cAC10 antibody drug conjugates (ADCs) with two and four MMAE molecules per antibody, C8-E2 and C8-E4 respectively, has been described (Hamblett et al., Clin. Cancer Res. 15 7063-7070 (2004)). Briefly, the method involved a partial reduction of the mAb to expose ~4 reduced Cys per Ab followed by reaction with

vc-MMAE. Partially loaded cAC10-Val-Cit-MMAE referred to as C8-E4-Mixture (or C8-E4M) was obtained.

C8-E2 and C8-E4 were prepared from C8-E4M by preparative HIC (hydrophobic interaction chromatography) fractionation on a Toyopearl Phenyl-650M HIC resin (Tosoh Bioscience, Montgomeryville, PA) equilibrated with >5 column volumes of Buffer A (50 mM sodium phosphate, 2 M NaCl, pH 7.0). To prepare the sample for loading onto the column, 39 ml of the C8-E4-Mixture (12.9 mg/ml) was mixed with an equivalent volume of Buffer A' (50 mM sodium phosphate, 4 M NaCl, pH 7.0). Following sample loading, the column was washed with Buffer A until an A_{280} baseline was achieved. C8-E2 was eluted and collected with a step gradient consisting of 65% Buffer A / 35% Buffer B (80% v/v 50 mM sodium phosphate, pH 7.0, 20% v/v acetonitrile). After baseline was again achieved, C8-E4 was eluted and collected with a step gradient consisting of 30% Buffer A / 70% Buffer B. Both C8-E2 and C8-E4 peaks were collected to ~20% of their respective peak heights. The fractions of interest were buffer exchanged into PBS using Ultrafree-15 centrifugal filter devices with a molecular weight cutoff of 30 kDa (Millipore, Billerica, MA).

(ii) Analysis of Drug Loading

Drug loading was determined by measuring the ratio of the absorbance at 250 and 280 nm (A250/280). The number of vcMMAE per cAC10 was empirically determined to be (A250/A280 – 0.36)/0.0686. ADCs were analyzed by hydrophobic interaction chromatography (HIC) using a Tosoh Bioscience Ether-5PW column (part 08641) at a flow rate of 1 mL/min and a column temperature of 30 °C. Solvent A was 50 mM sodium phosphate pH 7 and 2.5 M NaCl. Solvent B was 80% 50 mM sodium phosphate pH 7, 10% 2-propanol, and 10% acetonitrile. Isocratic 0% B for 15 min, a 50-min linear gradient from 0 to 100% B, a 0.1-min linear gradient from 100 to 0% B, and isocratic 0% B for 14.9 min. Injections (typically 90-100 µL) were 1 volume of ADC (concentration of at least 3 mg/mL) and 1 volume of 5 M NaCl.

ADCs from HIC chromatography were analyzed using an Agilent Bioanalyzer. A protein 200 chip was used under denaturing but nonreducing conditions as described by the manufacturer. Briefly, 4 µL of 1 mg/mL ADC were mixed with 2 µL of nonreducing

loading buffer and heated to 100 °C for 5 min. Water (84 µL) was added and 6 µL of this mixture was loaded into each well of the chip.

ADCs were analyzed on a PLRP-S column (Polymer Laboratories part 1912-1802: 1000 A, 8 u, 2.1x50 mm). The flow rate was 1 mL/min and the column temperature was 80 °C. Solvent A was 0.05% trifluoroacetic acid in water and solvent B was 0.04% trifluoroacetic acid in acetonitrile. Isocratic 25% B for 3 min, a 15-min linear gradient to 50% B, a 2-min linear gradient to 95% B, a 1-min linear gradient to 25% B, and isocratic 25% B for 2 min. Injections were 10-20 µL of 1 mg/mL ADC previously reduced with 20 mM DTT at 37 °C for 15 min to cleave the remaining interchain disulfides.

Results

MMAE conjugates of cAC10 cysteine variants were generated by reduction of the antibody followed by alkylation with vcMMAE. Analysis of each conjugated cAC10 cysteine variant by both UV-VIS analysis at an absorbance of 280 nm and PLRP chromatography demonstrated that close to the expected drug loading was achieved as shown in Table 4.

Table 4
Summary of the analysis of cAC10 cysteine variant vcMMAE conjugates

cAC10 Variant	Conc. (mg/ml)	Volume (ml)	Total (mg)	DTNB RSH/Ab	PLRP Drug/ab	% monomer	Free drug
C2v1-E2	41	2.3	94.3	2.3	2.1	98.0%	<0.05%
C2v2-E2	5.6	0.6	3.4	2.3	2.0	99.4%	<0.05%
C4v1-E4	12.0	3.8	45.6	4.2	3.7	99.1%	<0.05%
C4v2-E4	12.4	4.0	49.6	4.0	3.5	98.7%	<0.05%
C4v3-E4	0.8	0.4	0.3	7.2	3.7	99%	<0.05%
C6v1-E6	12.5	3.3	40.6	5.7	5.7	98.7%	<0.05%

Analysis by size exclusion chromatography demonstrated that all conjugates consisted of 98% monomer or greater as shown in Table 4. The control molecules described in this study were parent cAC10 conjugated with either two molecules of MMAE (C8-E2), or four molecules of MMAE (C8-E4). These two and four drug-loaded MMAE conjugates were generated by partial reduction of the parent cAC10 antibody and analyzed as previously described in Hamblett et al., Clin. Cancer Res. 15:7063-7070 (2004).

In vitro cytotoxicity of cAC10 cysteine variant conjugates

Procedures

Growth inhibition of CD 30^+ Karpas 299 cells treated with cAC10 cysteine variant conjugates was determined by measuring DNA synthesis. Conjugates were incubated with cells for 92 hours followed by labeling with [^{3}H]-thymidine, 0.5 μCi/well for 4 hours at 37 °C. Cells were harvested onto filters and mixed with scintillation fluid and radioactivity was measured using a Topcount Scintillation counter (Packard Instruments, Meriden, CT). The percent radioactivity incorporated relative to the untreated controls was plotted versus conjugate concentration and the data were fit to a sigmoidal dose-response curve using Prism 4 software (GraphPad Software Inc, San Diego, CA). Alternatively, 50 μM resazurin was added to Karpas 299 cells following the 92 hour incubation period with conjugate. After a 4 hour incubation period dye reduction was measured using a Fusion HT fluorescent plate reader (Packard Instruments, Meriden, CT).

Results

The cytotoxicities of the AC10 cysteine variant C2v1, C4v1, C4v2, and C6v1 MMAE conjugates (C2v1-E2, C4v1-E4, C4v2-E4, and C6v1-E6, respectively) were tested using a [^{3}H]-thymidine incorporation assay on $CD30^+$ Karpas 299 cells. The control conjugate used was the four drug-loaded parent cAC10 (C8-E4) which has been shown to have potency that lies between the fully loaded parent cAC10 MMAE conjugate (C8-E8) (which is the most potent), and the two-drug loaded conjugate (C8-E2). C6v1-E6 had the lowest IC_{50} value of 0.012 nM, while the four drug-loaded cysteine variants C4v1-E4 and C4v2-E4 and the four drug-loaded parent cAC10 conjugate C8-E4 had very similar IC_{50}s of 0.020 nM, 0.027 nM and 0.018 nM, respectively, as shown in Figure 2A. As shown in Figure 2B, the C2v1-E2 MMAE conjugate had an IC_{50} of 0.029 nM. Subsequently, the in vitro cytotoxic activities of both C2v1-E2 and C2v2-E2 MMAE drug conjugates on Karpas 299 cells were evaluated. Cytotoxicity was assessed by reduction of resazurin dye which was introduced to the culture following 92 hours continuous exposure to conjugate. cAC10 conjugated with two MMAE drug molecules per antibody (C8-E2) was used as the control. All three conjugates had similar IC_{50} values of 52.4 ng/ml, 39.8 ng/ml and 39.8 ng/ml for C2v1-E2, C2v2-E2 and C8-E2, respectively. These data demonstrate that the cysteine variant conjugates compare closely in activity to partially loaded MMAE conjugates generated from the parent cAC10 antibody by partial reduction.

Antitumor activity of cAC10 cysteine variant conjugates in vivo using a Xenograft model of human ALCL

Procedures

To establish a subcutaneous disease model of ALCL $5x10^6$ Karpas-299 cells were implanted into the right flank of C.B-17 SCID mice (Harlan, Indianapolis, IN). Therapy with ADCs was initiated when the tumor size in each group of 6-10 animals averaged 100 mm^3. Treatment consisted of a single injection. Tumor volume was calculated using the formula (length x $width^2$)/2. A tumor that decreased in size such that it was impalpable was defined as a complete regression (CR). A complete regression that lasted beyond 100 days post tumor implant was defined as a cure. Animals were euthanized when tumor volumes reached approximately 1000 mm^3.

Results

The efficacies of the cAC10 cysteine variant drug conjugates were assessed in a subcutaneous xenograft model of ALCL in SCID mice. Karpas 299 cells were implanted into the flanks of SCID mice and tumors were grown to an average volume of 100 mm^3. Tumor bearing mice were randomly divided into groups of eight to ten animals and either left untreated or were treated with cAC10 cysteine variant MMAE conjugates C2v1-E2, C4v1-E4 or C4v2-E4 or partially MMAE loaded parent cAC10 conjugates C8-E2 and C8-E4 in a single dose study. ADC doses were normalized so an equal concentration of MMAE was injected per group with 1 mg/kg, 1.14 mg/kg and 1.05 mg/kg injected for C8-E4, C4v2-E4 and C4v1-E4, respectively, and 2 mg/kg and 1.9 mg/kg for C8-E2 and C2v1-E2, respectively. As shown in Figure 3A, C2v1-E2 showed similar antitumor activity to C8-E2 with complete tumor regressions occurring in all animals treated with C8-E2 and six of eight animals treated with C2v1-E2. As shown in Figure 3B, C4v1-E4 and C4v2-E4 displayed similar antitumor activities to C8-E4. Complete regressions occurred in eight of ten animals for C8-E4 and C4v2-E4 and six of ten animals for C4v1-E4.

In summary, the two and four drug loaded ADCs generated from the cysteine variants have similar in vivo activity to the C8-E4 and C8-E2 conjugates produced by the partial reduction method.

Example 2

Preparation and Analysis of Antibody Conjugates