

Fusion proteins as biopharmaceuticals – Applications and challenges

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As the genetic combination of originally separate proteins, fusion proteins are successful biopharmaceuticals. This review summarizes the fusion proteins that are approved for use in the clinic and those that are currently in clinical trials. Fusion proteins can be categorized into several groups according to their features. In the first group, effector molecules are fused to Fc domains, albumin or transferrin to extend the plasma half-life of the fusion product. In the second group, toxicity is conveyed by fusion proteins to toxins, enzymes or cytokines. The third application, which is not yet in clinical trials, utilizes fusion partners to enable novel delivery and targeting routes. Besides some specific disadvantages, many examples of fusion proteins suffer from the challenge of immunogenicity; however, future applications with novel fusion partners will reach beyond cancer, immunology and inflammation.

Keywords Bi-functional protein, chimeric protein, fusion protein, immunoadhesin, immunotoxin

Abbreviations

ADEPT antibody-directed enzyme prodrug therapy, **EF** elongation factor, **Fc** fragment crystallizable, **FcRn** neonatal Fc receptor, **Ig** immunoglobulin, **PE** *Pseudomonas* exotoxin

Introduction

Therapeutic proteins have played a significant role against many diseases for more than 25 years. The first recombinant protein, insulin, was approved in 1982. Since then, the number of recombinant products has grown and the number of biopharmaceuticals approved as blood products, hormones, cytokines and monoclonal antibodies has increased considerably [1,2]. However, the impact of recombinant DNA technology reaches beyond the reproduction of natural proteins. For the first time, this technology has enabled the design of novel molecules that do not occur in nature. Typical examples are fusion proteins that are created by combining genes originally coding for separate proteins. The novel single polypeptide that is produced displays the functional properties of both parent molecules.

The focus of this review is the current status of therapeutic fusion proteins that were either approved or in clinical trials in 2008. A discussion of chemical conjugates, chimeric or bi-specific antibodies and multi-epitope vaccines is excluded and neither naturally occurring fusion proteins, as observed in several malignant cancers, nor various fusion tags to improve protein expression, are included in this review.

Fusion proteins can be categorized according to their incorporated protein domains. Frequently, one fusion partner has a molecular recognition function whereas

the other partner transfers certain functionality such as improvement of stability and half-life, cytotoxic effects or novel targeting and delivery routes. Therapeutic fusion proteins can be immunotoxins [3], cytokine fusions [4], fragment crystallizable (Fc)-fusions [5], albumin fusions [6] and others not belonging to any of these classes.

Although most of the ongoing clinical trials of recombinant proteins involve monoclonal antibodies, it is anticipated that fusion proteins will continue to be important therapeutic molecules in the future. In 2007, fusion proteins belonged to the top four most lucrative biotech sectors. The combined sales of four products reached US \$3.7 billion [7].

Current status of fusion protein therapeutics

There are currently six fusion proteins that have been approved and launched, shown in Table 1. Etanercept, which has the broadest range of applications, has been marketed since 1998 and is the most successful fusion protein with the highest sales figures to date [8]. This agent consists of two molecules of the 75 kDa fragment of the human TNF α receptor fused to the Fc part of immunoglobulin (Ig)G1. Etanercept reversibly binds TNF α , preventing TNF-dependent inflammatory diseases [9].

Denileukin difitox was launched shortly after etanercept and is currently the only approved therapeutic immunotoxin. In this pharmaceutical, human IL-2 is fused to the catalytic and transmembrane domains of native diphtheria toxin. Denileukin difitox targets malignant cells expressing the p55 (CD25) component of the IL-2 receptor, which is commonly expressed in cutaneous T-cell lymphoma [10]. The mechanism of action of this agent is described in Table 1.

Table 1. Selected approved therapeutic fusion proteins.

Drug	Launch	Company	Disease	Mechanism of action	Target	Protein 1	Protein 2	Reference
Etanercept (Enbrel)	November 1998	Amgen Inc	Ankylosing spondylitis, juvenile rheumatoid arthritis, plaque psoriasis, psoriatic arthritis and rheumatoid arthritis	TNF inhibitors	TNF α , TNF β	p75 TNF α R	IgG1 Fc	[9]
Denileukin diftitox (Ontak)	February 1999	Eisai Co Ltd	Cutaneous T-cell lymphoma	Inhibits activated T-lymphocytes expressing IL-2 receptors	IL-2 receptor	IL-2	Diphtheria toxin	[10]
Alefacept (Amevive)	January 2003	Astellas Pharma Inc/LG Life Sciences Ltd	Psoriasis	T-cell activation inhibitors	CD2	Human leukocyte function antigen-3 (LFA-3)	IgG1 Fc	[11]
Abatacept (Orencia)	February 2006	Bristol-Myers Squibb Co	Rheumatoid arthritis and juvenile rheumatoid arthritis	B7-dependent stimulation of T-cell proliferation inhibitors	CD80, CD86	Cytotoxic T-lymphocyte antigen-4 (CTLA-4)	IgG1 Fc	[12]
Rilonacept (Arcalyst)	February 2008	Regeneron Pharmaceuticals Inc	Cryopyrin-associated periodic syndromes	IL-1 inhibitors	IL-1	Ligand-binding domains of IL-1 receptor and IL-1 receptor accessory protein (IL-1RAcP)	IgG1 Fc	[13]
Romiplostim (Nplate)	August 2008	Amgen Inc	Immune thrombocytopenic purpura	Platelet biosynthesis stimulants	TPO receptor (Mpl)	Peptide	IgG1 Fc	[14]

CD cluster of differentiation, **Fc** fragment crystallizable, **TPO** thrombopoietin

In 2003, alefacept was approved as a treatment for psoriasis. This immunosuppressive dimeric fusion protein consists of the extracellular CD2-binding portion of the human leukocyte function antigen-3 (LFA-3) linked to the Fc domain of human IgG1. Alefacept induces apoptosis of activated memory effector T-cells by binding to CD2, preventing the LFA-3/CD2 interaction. Alefacept probably also disturbs the activation of other immune cells because CD2 can also be found on natural killer cells and certain bone marrow B-cells [11].

Abatacept consists of the human cytotoxic T-lymphocyte antigen-4 (CTLA-4) fused to an IgG1 Fc domain. This agent prevents T-cell proliferation by binding to CD80 (B7, B7-1) with high affinity. The CD80 receptor is located adjacent to the T-cell receptor on antigen-presenting cells and co-stimulates T-cell activation by binding to CD28. Currently, abatacept is approved as a treatment for rheumatoid arthritis and is in clinical trials for a series of other indications [12].

Rilonacept, the so-called IL-1 trap, became available on the market in 2008. This pharmaceutical is composed of the ligand-binding domains of IL-1 receptor and IL-1 receptor accessory protein (IL-1RAcP) fused to an IgG1 Fc domain. The receptor domains bind IL-1 α , and IL-1 β with reduced affinity. This prevents inflammation mediated through the activation of cell surface receptors. Rilonacept is currently approved for the long-term treatment of cryopyrin-associated periodic syndromes [13].

The second fusion protein to be launched in 2008 was romiplostin. This peptibody consists of a small bioactive peptide that is coupled to an IgG1 Fc domain to extend the plasma half-life of the protein. The peptidic part binds and activates the thrombopoietin (TPO) receptor (Mpl). Stimulation of Mpl sustains platelet counts and activates growth and maturation of bone marrow cells. Currently, romiplostin is approved as a therapy for chronic immune thrombocytopenic purpura [14].

Strategies to increase protein half-life

Many effector proteins are relatively small molecules that have short serum half-lives. The fast removal of those proteins from the blood stream narrows their therapeutic window and means they would require frequent administration. To overcome this limitation, several strategies have been employed including the fusion of the small effector protein or peptide to larger abundant proteins such as the Fc domains of immunoglobulins, albumin and transferrin.

Fc fusions

The antibody-combining region (Fab) is replaced by an effector molecule in Fc-fusions. The remaining constant (Fc) and hinge regions are sufficient to provide immune functions and to extend the half-life of the fusion protein.

Fc receptors

Half-life extension through Fc-fusion can be achieved by increasing the molecular weight, utilizing the different binding affinities of Ig isotypes to the Fc receptors or recycling via the neonatal Fc receptor (FcRn). FcRn expression in epithelial cells lining the intestine, lung and kidney remains relatively constant throughout life. In FcRn-dependent recycling, Fc-proteins are internalized through pinocytosis and interact with the FcRn within acidic endosomes. Fc-fusion proteins bound to FcRn are then shuttled back to the plasma membrane of the endothelial cells and released back into the circulation in a pH-dependent manner [15].

Cytotoxic effects

Toxicity can be conveyed by the Fc domain through mechanisms of antibody-dependent cellular cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). The cytotoxic potential of three anti-TNF α agents was recently compared. The antibodies infliximab and adalimumab and the fusion protein etanercept all contain the CH2 domain of the Fc portion of IgG1, exerting almost equal ADCC activities. The CH2 domain is recognized by Fc γ receptors on the surface of immune effector cells that steer the functions of phagocytosis or lysis; however, only the antibodies demonstrated a potent CDC effect because etanercept lacks the CH1 domain and hinge region that are required for CDC [16].

Fc-fusion proteins in clinical development

Many novel Fc-fusion proteins are now in clinical trials, as summarized in Table 2. Three fusion partners can be differentiated: ligands, peptides and receptor domains.

Approaches that have been employed to extend protein half-life include the very successful drugs erythropoietin and Factor IX (FXFc; Syntonix Pharmaceuticals Inc/Biovitrum AB). Interestingly, a later pulmonary application of the Factor IX Fc-fusion might be possible, taking advantage of the FcRn transport [5]. The leukocyte protein P-selectin glycoprotein ligand-1 is fused to IgG1 to block P-selectin. This prevents leukocytes and platelets from adhering and causing inappropriate inflammation and/or thrombosis, particularly in transplantations [17].

A technology called Mimetibody, developed by Centocor Inc, is the basis for CNTO-528. Here, a small hematopoietic peptide is fused to the Fc domain of IgG1, which stimulates erythropoiesis *in vivo* [18]. Amgen Inc has evaluated a similar concept with the development of peptibodies, which are small peptide antagonists fused to Fc in order to be protected from degradation and fast renal clearance. One candidate drug, A-623 (AMG-386; Anthera Pharmaceuticals Inc), acts as a B-cell inhibitor, whereas the other candidate, AMG-386 (Amgen Inc), inhibits angiopoietin and is being tested against various cancer indications in phase II clinical studies [19].

The largest group of Fc-fusions comprises the extracellular domain of a receptor as the effector molecule. One example, which uses the fibroblast growth factor receptor (FGFR) 1c as the Fc-fusion molecule, stops tumor progression by efficiently preventing all FGFR1 ligands from binding to any of the FGF receptors [20].

Acceleron Pharma Inc is evaluating Fc-fusions of the activin receptor IIA or IIB. By binding and blocking activin, bone resorption is stopped and bone formation is activated [21].

Atacicept (Merck Serono Sa) is in phase III clinical trials against various autoimmune disorders. The agent inhibits B-cell growth and the production of auto-antibodies by binding the cytokines APRIL and BLyS with the cytokine receptor (TACI) fused to Fc [22]. Aflibercept (Regeneron Pharmaceuticals Inc/sanofi-aventis) combines the second Ig domain of vascular epithelial growth factor receptor (VEGFR) 1 and the third Ig domain of VEGFR2. This agent binds all VEGF-A isoforms as well as placental growth factor, herefore inhibiting angiogenesis. Aflibercept follows the same 'trap' principle as rilonacept, efficiently blocking signaling [23]. A final fusion protein in phase III clinical trials is belatacept (Bristol-Myers Squibb Co), which is an improved variant of abatacept. Belatacept has a higher binding affinity for B7-1 and B7-2 than abatacept and is 10-fold more potent than abatacept at inhibiting some T-cell responses [24].

Advantages of Fc-fusions

Several features of the Fc domain make this molecule a highly preferred fusion partner. The Fc domain helps to solubilize hydrophobic proteins and extends the half-life of proteins by increasing their size and by FcRn-mediated recycling. The Fc domain offers increased avidity for multivalent ligands because of the bivalency of dimeric fusion proteins. Secondary cytotoxic functions can be incorporated by a careful selection of the Fc isotype. Secretion into the culture medium simplifies the downstream processing by the same established methods as for antibodies.

Disadvantages of Fc-fusions

The production of Fc-fusions is relatively expensive because eukaryotic expression systems are required to enable the disulfide bridge in the Fc-dimer to form properly. The size of dimeric fusion proteins might decrease the diffusion rate through mucus; however, this can be ameliorated by creating monomeric Fc-fusion proteins.

Table 2. Selected Fc-fusion proteins in clinical development.

	Phase	Name(s)	Company	Disease	Mechanism of action	Binder	Reference
	II	Recombinant Factor IX-FC (FIXFc)	Syntonix Pharmaceuticals Inc/ Biovitrum SA	Hemophilia B	Factor IX stimulants	Factor IX	[5]
	II	YSPSL	Y's Therapeutics Co Ltd	Deep vein thrombosis and delayed graft function	Selectin antagonists	Extracellular domain of P-selectin glycoprotein ligand-1	[17]
	I	A-623 (AMG 623)	Anthera Pharmaceuticals Inc	Systemic lupus erythematosus	B-cell inhibitors	Peptibody antagonist of B-cell activating factor	[47]
	II	AMG-386	Amgen Inc	Cancer	Angiopoietin-2 inhibitors	Peptibody that selectively inhibits angiopoietin-2	[19]
Receptor domains	I	FP-1039	FivePrime Therapeutics Inc	Solid tumors	Fibroblast growth factor type 1 receptor antagonists	Extracellular domain of human fibroblast growth factor receptor 1c (FGFR1)	[20]
	II	ACE-011 (ACTRIIA-IgG2-Fc), ACE-031 (ACTRIIB-IgG1)	Accelaron Pharma Inc/Celgene Corp	Anemia and musculoskeletal disorders	Activin inhibitors, bone formation stimulants and bone resorption inhibitors	Extracellular domain of human type II activin receptor IIA (ActRIIB in ACE-031)	[21]
	III	Atacicept	Merck Serono SA	Chronic lymphocytic leukemia and rheumatoid arthritis	B-cell inhibitors	Cytokine receptor (TACI)	[22]
	III	Aflibercept	Regeneron Pharmaceuticals/ sanofi-aventis	Cancer, diabetes and eye disorders	Growth factor inhibitors and antagonists	Second Ig domain of VEGFR1 and the third Ig domain of VEGFR2	[23]
	III	Belatacept	Bristol-Myers Squibb Co	Liver transplant rejection and renal transplant rejection	Immunosuppressants	Mutated cytotoxic T-lymphocyte antigen-4 (CTLA-4)	[24]

Albumin fusions

A different approach to prolonging serum half-life is fusion to albumin, which is the most abundant human plasma protein (35 to 50 g/l in human serum), having an average half-life of 19 days [25].

The Albumin Fusion Technology that has been developed by Human Genome Sciences Inc extends the half-life and stability and reduces the immunogenicity of effector proteins. Albumin can be fused to either the N- or C-terminus of the effector protein to avoid changing its biological activity. The most advanced product of this type is Albuferon α -2b (Novartis AG), which is currently in phase III clinical studies against hepatitis C, and which might replace the standard therapy with IFN α -2b because the latter has a half-life of only 2 to 3 h. Albuferon α -2b is even superior to the various PEGylated variants [26].

Further albumin fusion proteins are summarized in Table 3. The effector proteins are either hormones, growth factors, cytokines or peptides. Interestingly, there is a preference for agonistic effects as there are currently no 'trap' variants in clinical trials that include the binding features of extracellular receptor domains.

Advantages of albumin fusions

Fusing other proteins to albumin increases their bioavailability and half-life, and dosing is simplified, as only monomeric fusions are possible. Recombinant human albumin fusion proteins can be manufactured efficiently with yeast on a large scale and at typically lower costs than other methods of generating long-acting biopharmaceuticals. Blocking of active N- or C-termini of the fusion partners can be avoided because albumin can be fused at each end.

Disadvantages of albumin fusions

Albumin only acts as a stabilizing agent and does not offer any secondary functions such as cytotoxicity.

Transferrin fusions

The third potential fusion partner that can be used to increase half-life is transferrin. Although this technology is not as mature as Fc or albumin fusions, it could have a huge potential. A preclinical program based on a naturally occurring non-glycosylated form of transferrin was conducted by Biorex Pharmaceuticals Corp before it was acquired by Pfizer Inc in 2007. At that time, the preclinical portfolio consisted of glucagon-like peptide

Table 3. Selected albumin fusion proteins in preclinical and clinical development.

Development status	Name(s)	Company	Disease	Mechanism of action	Effector	Reference
Discovery	Albulin-G	Teva Pharmaceutical Industries	Type 1 and 2 diabetes mellitus	Insulins and analogs	Insulin	[48]
II	Cardeva	Teva Pharmaceutical Industries	Chronic heart failure	Diuretics	B-type natriuretic peptide	[49]
II	Albugranin (Neugranin)	Teva Pharmaceutical Industries	Neutropenia	Granulocyte colony-stimulating factor agonists	Granulocyte colony-stimulating factor	[50]
III	Albiglutide (Syncria)	GlaxoSmithKline plc	Type 2 diabetes mellitus	Glucagon-like peptide-1 agonists	Glucagon-like peptide-1	[51]
III	Albupheron α -2b	Novartis AG	Cancer, chronic myeloid leukemia and hepatitis C virus infection	IFN α stimulants	IFN α	[26]

and interferon- β as fusion partners. The advantages of transferrin are the low production costs in yeast and the flexibility of fusion to either the N- or C-terminus. Additionally, a completely new class of therapeutics could be developed by utilizing the surface loops of transferrin for peptide engineering. An interesting feature for further study is the ability of transferrin to cross the blood-brain barrier by receptor-mediated transcytosis.

Toxicity

Immunotoxins

The paradigm of the magic bullet, selectively binding and destroying only malignant cells, is the idealistic concept that led to the construction of toxin fusions. Initially, toxins were chemically conjugated to recognition molecules. This caused considerable problems with regard to reproducible stoichiometry between the binder and toxin, and involved labor-intensive production including steps for the coupling and removal of unbound toxins. This approach was soon replaced by a more elegant method of genetically linking the targeting molecule with the toxin molecule. However, this limits the choice of toxins to toxic proteins. In general, toxic proteins from two origins are currently applied. The first source is plant toxins, which are either class I ribosome-inactivating proteins (saporin, bouganin, gelonin, pokeweed antiviral protein [PAP] and bryodin 1) or class II ribosome-inactivating proteins (ricin, abrin, modeccin and mistletoe lectin). Plant toxins prevent the association of elongation factors (EFs) 1 and 2 with the 60s ribosome, thus inhibiting protein synthesis. One common problem is their nonspecific binding to mannose receptors on hepatocytes. This can be avoided by deglycosylation or mutagenesis of amino acids. There have been multiple constructs containing ricin-A, saporin, PAP and bryodin 1, but all of these were discontinued in clinical trials. Only a fusion with bouganin is currently in a phase I clinical study [27].

Another source of toxins is bacteria. The most frequently used bacterial toxins are *Pseudomonas* exotoxin (PE) and diphtheria toxin. Both these toxins inhibit EF2, thus disrupting protein synthesis. The bacterial toxins consist

of an enzymatic domain and a binding domain, which is replaced by other recognition molecules in the case of immunotoxins. The toxins are truncated in order to prevent unspecific binding. The variants of PE are PE40 and PE38, containing amino acids 253 to 613 (PE40) and 253 to 364 plus 381 to 613 (PE38). In the case of diphtheria toxin, DAB486 contains the first 486 amino acids and DAB389 contains the first 388 amino acids [28].

Recently, cytotoxic ribonucleases that are conjugated to targeting ligands have been tested in preclinical studies to circumvent the immunogenicity problem that is associated with bacterial and plant toxins [29].

Immunotoxins in clinical development

Currently, seven immunotoxins are in clinical trials targeting several cancer cells, as displayed in Table 4. Five of the fusion proteins utilize mutated and truncated variants of PE. The general principle of immunotoxins is the targeting of cell-surface tumor-associated antigens and their subsequent internalization by endocytosis. After cleavage in the endosome and translocation of the catalytic toxin domain to the cytosol, the EF is inhibited. The arrested protein synthesis then leads to cell death by inducing apoptosis [30].

Antibody fusions

The four antibody-based immunotoxins target receptors that are primarily expressed on cancer cells. The receptors of interest are the human epidermal growth factor receptor 2 (HER2), the CD22 receptor on B-cells and the epithelial cell adhesion molecule (EpCAM), which seems to be a pan-carcinoma antigen. A variant of the EpCAM antibody-based immunotoxin called Vicinium is applied topically using intravesical instillation to avoid immunogenicity problems.

Interleukin fusions

Other variants of immunotoxins rely on the specific binding of IL-3, -4 and -13 to their respective receptors on tumor cells. Interestingly, IL-13 binds to three different receptors (IL-4Ra, IL-13Ra1 and IL-13Ra2), therefore expanding

Table 4. Immunotoxins in clinical development.

	Phase	Name(s)	Company	Disease	Mechanism of action	Target	Binder	Toxin	Reference
Antibody fusion	I	Zemab	TopoTarget Germany AG	Cancer	HER2 inhibitors	ErbB2/HER2 receptor	Antibody against ERB2/HER2 receptor	<i>Pseudomonas</i> exotoxin	[52]
	I	CAT-8015 (GCR-8015)	MedImmune	Chronic lymphocytic leukemia, hairy cell leukemia and non-Hodgkin's lymphoma	CD22 antagonists and apoptosis stimulants	CD22 receptor on B-cells	dsFv, which targets the CD22 receptor	<i>Pseudomonas</i> exotoxin	[53]
	I	VB6-845	Viventia Biotech Inc	Solid tumors	Ribosomal protein inhibitors	EpCAM	Antibody against EpCAM	Bouganin	[54]
	II	Proxinium (VB4-845, Vicinium)	Viventia Biotech Inc	Bladder and head and neck cancer	Protein synthesis inhibitors	EpCAM	Antibody against EpCAM	<i>Pseudomonas</i> exotoxin	[55]
Interleukin fusion	I	SL-401	Stemline Therapeutics Inc	Acute myeloid leukemia	Protein synthesis inhibitors	IL-3R	IL-3	Diphtheria toxin	[56]
	II	DT388IL-3	Wake Forest University						
	II	PRX-321 (Interleukin-4-(38-37)-PE38KDEL)	Protox Therapeutics Inc	Glioblastoma and solid tumors	Apoptosis stimulants	IL-4R α and IL-13R α 1	IL-4	<i>Pseudomonas</i> exotoxin	[57]
	III	Cintredekin besudotox	NeoPharm Inc	Asthma, glioblastoma, glioma and pulmonary fibrosis	Apoptosis stimulants	IL-4R α , IL-13R α 1 and IL-13R α 2	IL-13	<i>Pseudomonas</i> exotoxin (PE38QQR)	[58]

CD cluster of differentiation, **EpCAM** epithelial cell adhesion molecule, **ErbB2/HER2** human epidermal growth factor receptor 2

the range of targeted cells. The application of cintredekin besudotox (Neo Pharm Inc) against glioblastoma is performed through convection-enhanced delivery (CED) where the drug is delivered through catheters inserted in brain tissue. This reduces the risk of immunogenicity and avoids problems associated with crossing the blood-brain barrier.

Advantages of immunotoxins

The biggest advantage of immunotoxins is the targeted toxicity that limits side effects. So far, immunotoxins have proved successful against hematological malignancies.

Disadvantages of immunotoxins

Frequently, low efficacy against solid tumors was observed, which is presumably because of the limited diffusion of the large molecules. Because 'non-human' toxic proteins are used, they are very immunogenic, which quickly leads to the development of anti-toxin antibodies. In addition, immunotoxins are usually difficult to produce because of their intrinsic cellular toxicity.

Cytokine fusions

A different class of toxic fusion proteins is those that combine targeting molecules with cytokines as effectors. These bi-functional molecules increase the tumor-killing activity of the antibodies and activate a secondary antitumor immune response by stimulating a broad variety of immune cells [31]. It has recently been confirmed that immunotherapy has the potential to destroy large tumors, achieving lasting remissions, and this approach is not confined to supportive treatment in patients with residual disease [4]. The primary application of cytokine fusions is to render some non-immunogenic tumors immunogenic, thus activating a protective immunity.

Cytokine fusions in clinical development

Currently, three different cytokines, IL-12, IL-2 and TNF α , are applied in clinical studies as displayed in Table 5. IL-2 is known to be a potent stimulator of the cellular and humoral immune systems and has huge therapeutic potential. IL-2 stimulates the growth and

proliferation of immune cells (T-cells, natural killer cells, lymphokine-activated killer cells and tumor infiltrating leukocytes) that destroy tumor cells [32].

IL-12 is a heterodimeric cytokine composed of p35 and p40 subunits that are encoded by two separate genes, *IL-12A* (p35) and *IL-12B* (p40). Only the heterodimer is active and functional. The immunomodulating and anti-angiogenic functions of IL-12 make it a promising anticancer agent [33].

TNF α has several effects on tumor-associated vasculature. This cytokine increases endothelial permeability, leading to improved chemotherapy penetration within the tumor tissue. Tumor vessels are destroyed through the selective killing of angiogenic endothelial cells: this is a dose-dependent process. At high doses, TNF α damages associated blood vessels leading to tumor necrosis. At low doses, the vascular permeability is increased, which improves the effectiveness of cytotoxic drugs in combination therapy [34].

All cytokine fusion proteins are applied in oncology indications and most are fused to antibodies against tumor cell surface antigens. In general, they act as immunostimulants by combining the antitumor activity of the antibody with stimulation of immune cells. Fusions with TNF α have anti-angiogenic effects. The cytokine fusion proteins are more effective than administering the separated proteins in combination.

Advantages of cytokine fusions

Cytokine fusion proteins concentrate the cytokine in the tumor microenvironment and increase the direct antitumor effect of the antibody without causing the severe toxic side effects of systemic high-dose cytokine administration. Another positive effect is the extension of the half-life by the same mechanisms as with other Fc-fusions.

Disadvantages of cytokine fusions

One disadvantage of cytokine fusions is the difference in effective concentrations that is required for the individual parts of the fusion protein. Cytokines are highly potent proteins, requiring a much lower dose than antibodies.

Antibody-directed enzyme prodrug therapy (ADEPT)

Targeted toxicity can also be conveyed by coupling an antibody with an enzyme that catalyzes the generation of a toxic product. This approach is called antibody-directed enzyme prodrug therapy (ADEPT). After administration and removal of unbound antibody-enzyme fusion protein, the prodrug is applied and then metabolized into a toxic compound. Despite numerous attempts since the mid 1980s, there is currently only one such fusion protein in phase I clinical trials [35]. In this fusion protein, a single chain variable domain antibody against carcinoembryonic antigen is fused to bacterial enzyme

carboxypeptidase G2. Bis-iodo phenol mustard is used as the prodrug [36].

Advantages of ADEPT

Much higher drug concentrations can be delivered to the cancer using ADEPT than by using any other targeted method. The small-molecule drug also demonstrates better diffusion into solid tumors. Even tumor cells that are not expressing the targeted antigen at sufficient concentration will be killed by the toxic metabolite through the so-called bystander effect [37].

Disadvantages of ADEPT

The many disadvantages of ADEPT have so far limited its clinical progress. The drug half-life is too long, which leads to the drug leaking out of the tumor and destroying healthy tissue. Enzymes of bacterial origin are frequently used in ADEPT to avoid the general toxicity associated with using endogenous human enzymes, but this in turn causes immunogenicity problems. Incomplete removal of unbound enzyme activity from the plasma can lead to unwanted toxicity in the whole organism [38].

Other fusion proteins in clinical development

In addition to the examples described above, there are several fusion proteins against various indications currently in clinical trials that do not fit into the categories described previously, shown in Table 6.

APO-010 (Topo Target A/S) consists of three human FasL extracellular domains linked to a protein backbone comprising the dimer-forming collagen domain of human adiponectin. This fusion protein binds to CD95 (Apo1 receptor) and induces caspase-dependent apoptosis [39]. In order to cure hypophosphatasia by enzyme replacement therapy, the catalytic domain of human tissue non-specific alkaline phosphatase is fused to a short peptide. This peptide targets the enzyme to bone tissue where it activates bone mineralization [40]. Another fusion protein, BA-210 (Alseres Pharmaceuticals Inc), inhibits Rho. Blocking of this kinase-signaling pathway contributes to axon regeneration and functional recovery of movement and sensation following spinal cord injury [41].

A more advanced fusion protein, naptumomab estafenatox (Active Biotech AB), applies the concept of tumor-targeted superantigens. In contrast to typical immunotoxins, the fusion between an antibody targeting the cancer-specific oncofetal protein 5T4 and *Staphylococcal* enterotoxin leads to the stimulation of T-cell response and indirect tumor killing [42].

The last of this group of fusion proteins, amediapase (The Menarini Group), is a drug against myocardial infarction. The dual functionality results from the kringle 2 domain of the tissue plasminogen activator fused to the carboxy terminal protease region of pro-urokinase. This combination of fibrinolytic and thrombolytic activity efficiently dissolves blood clots [43].

Table 5. Cytokine fusion proteins in clinical development.

Phase	Name	Company	Disease	Mechanism of action	Target	Binder	Cytokine	Reference
I	AS-1409 (BC1-IL-12)	Antisoma plc	Renal cancer and skin melanoma	Interferon type II stimulants	ED-B, a protein associated with tumor blood vessels	BC1 antibody against ED-B oncofetal fibronectin splice variant	IL-12	[59]
II	ALT-801 (STAR-Ck)	Altor BioScience Corp	Cancer	Immunostimulants and T-lymphocyte stimulants	p53-derived tumor-associated antigen	Three domain human leukocyte antigen (HLA-A2)-restricted T-cell receptor specific for the human p53 protein	IL-2	[60]
II	EMD-273063 (hu14.18-IL-2)	Merck Serono SA	Neuroblastoma, melanoma	Immunostimulants	GD2 ganglioside on tumor cells	Humanized anti-CD2 monoclonal antibody	IL-2	[61]
II	Tucotuzumab celmoleukin	Merck Serono SA	Ovarian cancer and prostate cancer	Immunostimulants	EpCAM	Monoclonal antibody binds to EpCAM	IL-2	[62]
II	L19-IL-2 fusion protein	Bayer Schering Pharma AG	Colon cancer, teratocarcinoma, small-cell lung cancer and other solid tumors	Angiogenesis inhibitors, immunostimulants and IL-2 receptor agonists	ED-B	L19 single chain antibody targeting the ED-B protein	IL-2	[63]
II	L19-TNF α	Bayer Schering Pharma AG	Cancer	Angiogenesis inhibitors, immunostimulants and TNF agonists	ED-B	L19 single chain antibody targeting the ED-B protein	TNF α	[64]
II	Arenegyr	MolMed SpA	Colorectal cancer, liver cancer and mesothelioma	Angiogenesis inhibitors and TNF stimulants	CD13 aminopeptidase-N	Tumor homing peptide (NGR and CNGRCG)	TNF α	[65]

CD cluster of differentiation, **ED-B** extra domain B fibronectin, **EpCAM** epithelial cell adhesion molecule

Challenges

Besides the inherent disadvantages of specific fusion constructs as summarized for each category, there are challenges that concern fusion proteins in general.

Immunogenicity

In the huge variability of fusion proteins, non-human proteins are often used, which elicit an immune response that can be a severe complication. However, the immunogenicity of these proteins can be minimized by eliminating T-cell epitopes, that is, peptides that bind to T-cell receptors or the MHC class II. Mutating these residues while maintaining biological activity can result in modified therapeutic proteins with decreased immunogenicity [44].

Beside the intrinsic sequence-dependent causes for an immune response, immunogenicity can also be the result of the incorrect handling of the protein. For example, the expression system could add unconventional sugar moieties, immunogenic aggregates can remain after downstream processing, and proteins that are denatured during formulation may be immunogenic. A properly designed manufacturing process takes all these parameters into account [45].

Production

The process used to manufacture fusion proteins is a critical step and this is particularly true for immunotoxins. Frequently, these immunotoxins are already toxic to cells during production or result in inclusion body formation when expressed in bacterial systems. Refolding is feasible, but the overall yields are usually low, limiting the productivity and increasing the cost of products.

Conclusion and future perspective

Fusion proteins are promising in terms of therapeutic spectrum, efficiency and safety. The combination of multiple cytokines or effector molecules on a single targeting antibody can enable a highly efficient immunotherapy that optimizes the primary immune response and eventually leads to permanent antitumor immunity [31]. Another area for future development is Fc domain engineering. As described above, Fc-fusions can have multiple effects and can be optimized for specific applications. Here, the sector of fusion proteins can benefit from the insight obtained through studying monoclonal antibodies and Fc-receptor interactions [46]. Other new developments might cover approaches for the intracorporal and intracellular delivery of therapeutic proteins, although no such fusion protein has yet entered clinical trials.

Table 6. Other fusion proteins in clinical development.

Phase	Name	Company	Disease	Mechanism of action	Target	Protein 1	Protein 2	Reference
I	APO-010	TopoTarget A/S	Hematological malignancies and solid tumors	Apoptosis stimulants	CD95 and Apo1 receptors	mega-Fas-ligand	Collagen domain of human adiponectin	[39]
II	ENB-0040	Enobia Pharma Inc	Hypo-phosphatasia	Enzyme stimulants	Bone tissue	Peptide used to target the enzyme to the bone	Catalytic domain of human tissue non-specific alkaline phosphatase (TNSALP)	[40]
II	BA-210	Alseres Pharmaceuticals Inc	Spinal cord injuries	Rho-associated kinase inhibitors	Rho kinase	Rho inhibitor recombinant protein	Fibrin	[41]
III	Naptumomab estafenatox (Anyara)	Active Biotech AB	Non-small cell lung cancer, pancreatic cancer and renal cancer	T-lymphocyte stimulants	5T4 antigen	Antibody against the tumor-specific antigen 5T4	<i>Staphylococcal</i> enterotoxin A (SEA)	[42]
III	Amediplase	The Menarini Group	Myocardial infarction	Urokinase-type plasminogen activator stimulants	Fibrin and plasminogen	Kringles 2 domain from the A-chain of tissue plasminogen activator (t-PA)	Carboxy terminal region of pro-urokinase	[43]

CD cluster of differentiation

Fusion proteins are well-established therapeutic proteins, with successful drugs on the market and ongoing development of promising new molecules. Several typical fusion partners and categories can be distinguished. Fusion proteins are in constant competition with monoclonal antibodies, but offer certain advantages. These include the ability to directly transfer toxicity or recruit and stimulate the immune system to achieve tumor regression and immunity. Some fusion proteins represent improvements over naturally occurring proteins with regard to stability and effectiveness. The repertoire of combinations is very broad, addressing a huge variety of disease areas. Developers of fusion proteins can frequently benefit from experience obtained from the development of antibodies. This is particularly true when using antibodies or fragments as the targeting domain or when recruiting the Fc domain as the fusion partner. However, in contrast to monoclonal antibodies, there are only a few patents blocking the development of molecules. The freedom to operate is only limited by the biology. Here, the major challenge is to avoid or minimize immunogenicity. Further clinical success of the molecules discussed in this report will demonstrate the general applicability of fusion proteins beyond the well-established applications in oncology, immunology and inflammation.

Acknowledgements

The author would like to thank Maria Ersoy, Ulrika Ritzén and Esmeralda Woesteneken for many helpful discussions during the preparation of this manuscript.

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