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Therapeutic Proteins

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Abstract

Protein-based therapeutics are highly successful in clinic and currently enjoy unprecedented recognition of their potential. More than 100 genuine and similar number of modified therapeutic proteins are approved for clinical use in the European Union and the USA with 2010 sales of US \$108 bln; monoclonal antibodies (mAbs) accounted for almost half (48%) of the sales. Based on their pharmacological activity, they can be divided into five groups: (a) replacing a protein that is deficient or abnormal; (b) augmenting an existing pathway; (c) providing a novel function or activity; (d) interfering with a molecule or organism; and (e) delivering other compounds or proteins, such as a radionuclide, cytotoxic drug, or effector proteins. Therapeutic proteins can also be grouped based on their molecular types that include antibody-based drugs, Fc fusion proteins, anticoagulants, blood factors, bone morphogenetic proteins, engineered protein scaffolds, enzymes, growth factors, hormones, interferons, interleukins, and thrombolytics. They can also be classified based on their molecular mechanism of activity as (a) binding non-covalently to target, e.g., mAbs; (b) affecting covalent bonds, e.g., enzymes; and (c) exerting activity without specific interactions, e.g., serum albumin. Most protein therapeutics currently on the market are recombinant and hundreds of them are in clinical trials for therapy of cancers, immune disorders, infections, and other diseases. New engineered proteins, including bispecific mAbs and multispecific fusion proteins, mAbs conjugated with small molecule drugs, and proteins with optimized pharmacokinetics, are currently under development. However, in the last several decades, there are no conceptually new methodological developments comparable, e.g., to genetic engineering leading to the development of recombinant therapeutic proteins. It appears that a paradigm change in methodologies and understanding of mechanisms is needed to overcome major challenges, including resistance to therapy, access to targets, complexity of biological systems, and individual variations.

Keywords

Therapeutics; Proteins; Antibodies; Vaccines; Cancer; Immune diseases; Immunogenicity; Safety; Efficacy

1. Introduction

Proteins, e.g., albumin from egg whites, blood serum albumin, fibrin, and wheat gluten, were recognized in the eighteenth century as biological molecules with distinct properties mostly by their ability to coagulate under treatments with heat or acid. The term “protein” to describe these molecules was proposed in 1838 by Jöns Jakob Berzelius—from French

proteins and German Protein originated from Greek πρωτεῖος (primary) derived from πρῶτος (first, foremost, in time, place, order, or importance). Although one can argue which molecules are most important for life and they all are, currently the proteins as therapeutics are the most important biologicals in terms of their clinical utility. Close to 100 genuine unmodified therapeutic proteins have been approved for clinical use in the European Union (EU) and the USA by July 2011; in contrast, we still have to wait for the first approved DNA-based therapeutic. In 2010, sales of mainly recombinant therapeutic proteins and antibodies exceeded US\$ 100 bln (from US\$ 92 bln in 2009 to US\$ 108 bln in 2010); therapeutic monoclonal antibodies (mAbs) accounted for almost half (48%) of the sales—the top five biologics in 2010 sales are four mAbs and one antibody (IgG1 Fc)-derived fusion protein (Table 1) (<http://www.lamerie.com/press-room/biologics-sales-2010-exceeded-us-100-bln.html>).

Therapeutic mAbs and other therapeutic proteins have been reviewed previously (see recent reviews (1–13) and articles cited there). Therefore, here I will only update and briefly overview currently approved therapeutic proteins, and will describe comparatively therapeutic mAbs as the fastest growing groups of protein therapeutics to illustrate properties, challenges, and future directions that are common for all therapeutic proteins. I will also describe a new classification based on their molecular mechanism of activity.

We have suggested that there were two major paradigm changes in the development of therapeutic antibodies (9). The first occurred more than a century ago and resulted in the development of the serum therapy which saved thousands of lives; von Behring who in the 1880s developed an antitoxin that did not kill the bacteria but neutralized the toxin that the bacteria released into the body was awarded the first Nobel Prize in Medicine in 1901 for his role in the discovery and development of a serum therapy for diphtheria. The second major paradigm change began in the 1970s with the discovery of the hybridoma technology (14) which can provide unlimited quantities of mAbs with predefined specificity. The use of a number of molecular biology techniques, mostly recombinant DNA technology, and the increased understanding of the antibody structure and function led to the development of chimeric and humanized mAbs. Finally, phage display techniques and other techniques based on the progress of molecular biology, including the generation of transgenic animals, allowed the development of fully human antibodies which completed the paradigm change which occurred mostly during a period of two to three decades beginning in the 1970s and ending in the 1990s.

The revolutionary changes in science that resulted in the development of antibody therapeutics had broader implications for protein therapeutics in general. The first protein therapeutic other than antibodies, insulin, was purified from animal pancreases and administered to patients with diabetes mellitus in 1922 following the first paradigm change. The availability, cost, and immunogenicity of animal-derived insulin limited its use. It took 60 years and the second paradigm change in the 1970s to produce the first recombinant protein therapeutic, humulin (human insulin) (15). The second paradigm change for therapeutic proteins other than mAbs began with the development of recombinant DNA technologies in the 1970s, PCR, and other advancement in molecular biology, and ended in the 1990s. Interestingly, mAbs are unique among therapeutic proteins in that the hybridoma

technology independently on recombinant DNA and other molecular biology methodologies has been capable to provide single well-characterized species that have therapeutic potential. Indeed, the first therapeutic mAb approved for clinical use (1986, Table 1), Muromonab-CD3 (OKT3), is a murine mAb produced by hybridoma technology; it was withdrawn from the market and supplies were exhausted in 2010. Currently, therapeutic proteins are being gradually improved for efficacy, safety, quality, and cost and new targets are being explored, but there are no new concepts similar to those leading to the development of recombinant proteins and identification of unique high-affinity binders out of billions of different molecules.

Excluding protein-based vaccines and diagnostics which will not be discussed here, currently, there are more than 100 approved for clinical use genuine therapeutic proteins of which 29 are mAbs, 22 are enzymes, and the rest are of various structures and function. Based on their pharmacological activity, they can be divided into five groups: (a) replacing a protein that is deficient or abnormal; (b) augmenting an existing pathway; (c) providing a novel function or activity; (d) interfering with a molecule or organism; and (e) delivering other compounds or proteins, such as a radionuclide, cytotoxic drug, or effector proteins (8). Protein therapeutics can be also grouped based on their molecular types that include antibody-based drugs, anticoagulants, blood factors, bone morphogenetic proteins, engineered protein scaffolds, enzymes, Fc fusion proteins, growth factors, hormones, interferons, interleukins, and thrombolytics (5). Based on their molecular mechanism of function, they can be divided into three groups: (a) specific noncovalent binders, (b) proteins affecting covalent bonds, and (c) others. The first group of proteins approved by the European Union or the USA for clinical use by July 2011 consists of 73 genuine unmodified proteins including 29 mAbs (Tables 2, 3, and 4), the second—22 including 21 enzymes (Table 5); and the third group has only one representative, human serum albumin, which is used to increase plasma osmolarity. One should note that there is a group of polyclonal antibodies (either nonspecific pooled human immunoglobulin (Ig) or specific Ig) which are still in clinical use against toxins (diphtheria, tetanus, botulism), viruses (hepatitis A, hepatitis B, cytomegalovirus, varicella zoster, rabies, measles, vaccinia), venom toxins, and toxic drugs (digoxin); nonspecific pooled human IgG is also approved for use against idiopathic thrombocytopenic purpura, Kawasaki disease, and IgG deficiency. If these Igs are included as therapeutic proteins, then the total number of genuine therapeutic proteins exceeds 100. The largest and currently most selling therapeutic protein group is the first one and especially mAbs and Fc fusion proteins, including the top six-selling protein therapeutics in 2010 (Table 1).

2. mAbs and Fc Fusion Proteins

2.1. mAbs and Fc Fusion Proteins Approved for Clinical Use or in Clinical Trials

Currently (as of July 2011) 29 mAbs are approved for clinical use in the European Union or the USA (Table 2) and 6 Fc fusion proteins (Table 3). Four of the approved mAbs were withdrawn from the market for safety or utility reasons. One of the mAbs, Synagis, is for prevention and not for therapy but traditionally is included as a therapeutic mAb. Worldwide sales of mAb-based drugs in 2010 were \$52 billion of \$108 billion total for all protein

biopharmaceuticals; six of the ten top-selling therapeutic proteins in 2010 were mAbs (infliximab, bevacizumab, rituximab, adalimumab, trastuzumab, and ranibizumab) and one (etanercept)—Fc fusion protein (Table 1).

One antibody drug conjugate (ADC), Brentuximab vedotin, is pending approval; if approved, it will be the first in class. It comprises an anti-CD30 mAb attached by a protease-cleavable linker to a potent, synthetic drug, monomethyl auristatin E (MMAE) utilizing Seattle Genetics' proprietary technology. The ADC employs a novel linker system that is designed to be stable in the bloodstream but to release MMAE upon internalization into CD30-expressing tumor cells. This approach is intended to spare non-targeted cells, which may help minimize the potential toxic effects of traditional chemotherapy while allowing for the selective targeting of CD30-expressing cancer cells, thus potentially enhancing the antitumor activity.

Hundreds of mAbs are in thousands of clinical trials including 25 (12) in phase 3 trials—2,909 entries for planned, ongoing or completed clinical trials were retrieved from <http://www.clinical-trials.gov> by searching with therapy AND mAbs as of July 2011 of which 484 are in phase 3. Significant number of all new medicines are mAbs (see also <http://www.phrma.org/research/new-medicines>). More than 200 different antibody-based candidate therapeutics are in clinical trials targeting more than 70 different molecules. At least one to three different antibodies are being developed at different companies for each relevant therapeutic target. However, some molecules are targeted by many more mAbs, e.g., the insulin-like growth factor receptor type I (IGF-IR) is targeted by more than ten different mAbs (16). During the last decade and especially in the last several years, the number of clinical trials with therapeutic antibodies has increased dramatically. However, this increase has been largely due to an increase in the number of indications for the same antibodies, especially in combination with other therapeutics. The number of targets and corresponding antibodies in preclinical development and in the discovery phase has also increased significantly during the past decade.

Second- and third-generation mAbs are being developed against already validated targets. For example, based on Synagis, an antibody (motavizumab—MEDI-524; NuMax) was developed with much higher affinity to the F protein of the RSV (17). The improvement of already existing antibodies also includes an increase (to a certain extent) of their binding to Fc receptors for enhancement of antibody-dependent cell-mediated cytotoxicity (ADCC) and half-life, selection of appropriate frameworks to increase stability and yield, decrease of immunogenicity by using in silico and in vitro methods, and conjugation to small molecules and various fusion proteins to enhance cytotoxicity. A major lesson from the current state of antibody-based therapeutics is that gradual improvement in the properties of existing antibodies and identification of novel antibodies and novel targets are likely to continue in the foreseeable future. This is likely to be a major driving force of the field until saturation is reached presumably in the next decade or two, and various combinations of antibodies and other drugs may dominate unless a major change in the current paradigm occurs. Currently, research and development (R&D) of mAbs as potential therapeutics is growing, and therapeutic mAbs are the fastest growing group of therapeutic proteins.

Antibodies are used not only as antigen binders but also as contributing effector functions through their Fc to already known binders. Currently approved fusion proteins are mostly Fc fusions (Table 3). The rationale to develop Fc fusion proteins is that Fc can confer effector functions and long half-life because of binding to the neonatal Fc receptor (FcRn) and increase in size. In addition, because Fc is dimeric, such fusion proteins could exhibit activity due to increase in valency. An Fc fusion protein (etanercept) continues to be the top-selling therapeutic protein in 2010 with worldwide sales of \$7.287 bln. It is a TNF α inhibitor and is effective only in its dimeric form due to the Fc which also confers long half-life in the circulation. Interestingly, two other TNF α inhibitors (adalimumab and infliximab) are also among the top five selling protein therapeutics; the total sales for these three therapeutics exceed \$20 bln which makes TNF α a molecule targeted by the highest-selling therapeutic proteins. Romiplostim is the first peptide-Fc fusion (“peptibody”) approved as a human therapeutic; it is a thrombopoietin (TPO) receptor agonist for treatment for thrombocytopenia. Two Fc fusion proteins (Abatacept and Belatacept) are fused to the same molecule (CTLA4) but are used for different indications—rheumatoid arthritis and for the prevention of acute rejection in adult patients who have had a kidney transplant, respectively.

2.2. Beyond Traditional Antibodies: Engineered Antibody Domains

One question is whether a new paradigm change could trigger a new dramatic expansion of some novel, still unknown, types of therapeutics as it happened several decades ago. We do not know the answer to this question and surprises are always possible, but currently there are no indications that another paradigm change in the discovery of biological therapeutics is coming anytime soon (one methodology which could contribute to such paradigm change is the revolution in high-throughput sequencing but time will show how useful it is for development of therapeutic proteins). It rather appears that there will be gradual improvements of existing antibodies and identification of antibodies to novel targets using currently available methodologies. However, one area where one could expect conceptually novel antibody-based candidate therapeutics even though within the current paradigm is going beyond traditional antibody structures.

Currently, almost all FDA-approved therapeutic antibodies (Table 2) and the vast majority of those in clinical trials are full-size antibodies mostly in IgG1 format of about 150 kDa size. A fundamental problem for such large molecules is their poor penetration into tissues (e.g., solid tumors) and poor or absent binding to regions on the surface of some molecules (e.g., on the HIV envelope glycoprotein) which are accessible by molecules of smaller size. Therefore, a large amount of work especially during the last decade has been aimed at developing novel scaffolds of much smaller size and higher stability (see, e.g., recent reviews (9, 18, 19)). Such scaffolds are based on various human and nonhuman molecules of high stability and could be divided into two major groups for the purposes of this review—antibody derived and others. Here, I will briefly discuss advantages of antibody-derived scaffolds, specifically those derived from antibody domains, and binders selected from libraries based on engineered antibody domains (eAds); an excellent recent review describes the second group (18).

Firstly, their size (12–15 kDa) is about an order of magnitude smaller than the size of an IgG1 (about 150 kDa). The small size leads to relatively good penetration into tissues and the ability to bind into cavities or active sites of protein targets which may not be accessible to full-size antibodies. This could be particularly important for the development of therapeutics against rapidly mutating viruses, e.g., HIV. Because these viruses have evolved in humans to escape naturally occurring antibodies of large size, some of their surface regions which are critical for the viral life cycle may be vulnerable for targeting by molecules of smaller size including eAds. Secondly, eAds may be more stable than full-size antibodies in the circulation and can be relatively easily engineered to further increase their stability. For example, some eAds with increased stability could be taken orally or delivered via the pulmonary route or may even penetrate the blood-brain barrier, and retain activity even after being subjected to harsh conditions, such as freeze-drying or heat denaturation. In addition, eAds are typically monomeric, of high solubility, and do not significantly aggregate or can be engineered to reduce aggregation. Their half-life in the circulation can be relatively easily adjusted from minutes or hours to weeks by making fusion proteins of varying size and changing binding to the FcRn. In contrast to conventional antibodies, eAds are well expressed in bacterial, yeast, and mammalian cell systems. Finally, the small size of eAds allows for higher molar quantities per gram of product, which should provide a significant increase in potency per dose and reduction in overall manufacturing cost. However, in spite of all these advantages, there is still no candidate therapeutic based on such scaffolds in phase III clinical trial as of July 2011.

Research on novel antibody-derived scaffold continues. We identified a VH-based scaffold which is stable and highly soluble (20). It was used for construction of a large-size (20 billion clones) eAd phage library by grafting CDR3s and CDR2s from five of our other Fab libraries and randomly mutagenizing CDR1. Panning of this library with an HIV Env complexed with CD4 resulted in the identification of a very potent broadly cross-reactive eAd against HIV, m36, which neutralized primary HIV isolates from different clades with IC₅₀s and IC₉₀s in the low µg/ml range (21). Fc fusion proteins of m36 were even more potent and neutralized all tested isolates (22). I also proposed to use engineered antibody constant domains (CH2 of IgG, IgA, and IgD, and CH3 of IgE and IgM) as scaffolds for construction of libraries (23). Because of their small size and the domain role in antibody effector functions, these have been termed nanoantibodies, the smallest fragments that could be engineered to exhibit simultaneously antigen binding and effector functions. Several large libraries (up to 50 billion clones) were constructed and antigen-specific binders successfully identified (24). We have recently engineered CH2-based scaffolds with high stability by introducing an additional disulfide bond (25) and by shortening CH2 (26). It is possible that these and other novel scaffolds under development could provide new opportunities for identification of potentially useful therapeutics.

3. Therapeutic Proteins Other Than Antibody-Based

3.1. Therapeutic Proteins Approved for Clinical Use

Therapeutic proteins other than mAbs and Fc fusion proteins approved for clinical use by the US FDA include noncovalent binders (Table 4), proteins that affect covalent bonds which

are almost all enzymes (Table 5), and albumin. Based on their molecular type and similarity in function, they can be divided into anticoagulants, blood factors, bone morphogenetic proteins, engineered protein scaffolds, enzymes, growth factors, hormones, interferons, interleukins, and thrombolytics (5). There are several major differences and similarities between these proteins and antibody-based therapeutics which may explain why on average per therapeutic the approved mAbs and Fc fusion proteins are more successful in terms of sales. The Fc portion of the antibody confers them with relatively long half-life by binding to the FcRn and effector functions including ADCC and complement (27). There is no other protein capable of performing those functions simultaneously. A second major advantage is the ability of the Fabs to bind to large number of targets keeping their Ig-based scaffold. Attempts to design similar protein scaffolds based on other proteins are ongoing, but so far none has reached approval for clinical use. A third one is that antibodies have evolved to fight diseases and are in high concentrations (tens of mgs/ml) in the blood without significant side effects. Therefore, mAbs and Fc fusion proteins on average should be less toxic than other protein therapeutics.

3.2. Engineered Proteins to Resemble Fc Properties

Various methodologies have been used to engineer therapeutic proteins to resemble some of the properties which mAbs already have mostly through their Fc. To increase their half-lives, they are PEGylated (conjugated with PEG) which is currently the most successful, clinically and commercially, conjugation of proteins. An example of such protein is peginterferon- α 2b. There are currently several alternative competing conjugations with conformationally flexible stretches of amino acid residues, e.g., G, that lead to an increase in the hydrodynamic radius and correspondingly to an increase in the half-life of protein therapeutics. To confer cytotoxic functions, proteins can be conjugated to small molecule drugs and radionuclides similarly to antibodies.

3.3. Enzymes

Enzymes are a special class of protein therapeutics (Table 5). Although there are known antibodies with catalytic properties, typically they are not that active as highly specialized enzymes and currently all enzymes approved for clinical use are proteins different than antibodies. Interestingly, there is one protein approved for clinical use which changes a covalent bond but is not an enzyme—antithrombin III (AT-III). It inactivates thrombin by forming a covalent bond between the catalytic serine residue of thrombin and an arginine reactive site on AT-III.

4. Therapeutic Proteins: Successes and Challenges

The success of protein-based therapeutics is mostly due to the use of concepts and methodologies developed during the second paradigm change decades ago that resulted in dramatic improvement of three key features in candidate therapeutics required for FDA approval: safety, efficacy, and quality. They are critical for the success of any drug and are discussed in more detail below mostly with examples for antibody-based therapeutics which share similar challenges with other therapeutic proteins.

4.1. Safety

Side effects due to therapeutic proteins could be divided into two large groups: (a) interactions with intended targets and (b) interactions with unintended targets. Binding to an intended target can lead to undesirable side effects, e.g., by immunomodulatory antibodies that could be suppressory or stimulatory. Administration of suppressory therapeutic proteins could lead to wide range of side effects related to decreased function of the immune system. An important example is the use of the best-selling antibody-based protein therapeutics targeting TNF α (etanercept, infliximab, certolizumab pegol, and adalimumab) which can lead to infectious complications (28). The overstimulation of the immune system can also produce life-threatening illness. In one case which gained wide publicity, administration of a single dose of the stimulatory anti-CD28 mAb TGN1412 resulted in induction of a systemic inflammatory response characterized by a rapid induction of pro-inflammatory cytokines in all six volunteers, leading to critical illness in 12–16 h (29). One important difference between antibody-based therapeutic containing Fc and other therapeutic proteins (not conjugated with toxic molecules) is that the antibody effector functions including ADCC and complement-dependent cytotoxicity (CDC) could lead to toxicities after binding to intended target molecules but on tissues other than those intended. An example of this is the trastuzumab-associated cardiotoxicity that is potentiated when the antibody is used concurrently or sequentially with an anthracycline (30).

Interactions with unintended targets can lead to a wide range of side effects in many cases with poorly understood mechanisms. An important example is the adverse acute infusion reactions after administration of proteins, where cytokine release plays a pivotal role, but other not fully explained mechanisms could be involved; such reactions were reported for many proteins, including infliximab, rituximab, cetuximab, alemtuzumab, trastuzumab and panitumumab (31), insulin, and interferon. Infusion side effects for rituximab can result from release of cellular contents from lysed malignant B cells (32). Administration of proteins can also lead to hypersensitivity reactions, including anaphylactic shock and serum sickness (28). Preexisting IgEs that cross-react with therapeutic proteins can increase the number and severity of such reactions, which can occur even with the first protein infusion. A notable example of this occurred with administration of cetuximab (31). Hypersensitivity is frequently associated with immunogenicity.

4.2. Immunogenicity

Immunogenicity of proteins can be a significant safety and efficacy issue (28, 33–38). For example, the success of the mAb-based therapeutics was critically related to the development of less immunogenic proteins. Murine mAbs were used initially as candidate therapeutics in the 1980s, but their high immunogenicity resulted in high titers of human anti-mouse antibodies (HAMAs), and related toxicities and low potency. Development of the less immunogenic chimeric mAbs, which contain human Fc fragments, and humanized mAbs, which contain mouse complementarity determining regions (CDRs) grafted into human antibody framework, was critical for the clinical success of the products. Human antibodies exhibit low immunogenicity on average, and are currently the favored type of antibody in development, although most of the therapeutic antibodies approved for clinical use are still chimeric and humanized mAbs.

Immunogenicity can be influenced by factors related to protein structure, composition, posttranslational modifications, impurities, heterogeneity, aggregate formation, degradation, formulation, storage conditions, as well as properties of its interacting partner, the patient's immune system and disease status, concomitant medications, dose, route, and time and frequency of administration especially when administered as multiple doses over prolonged periods (34). Even human proteins can elicit human anti-human antibodies. In one of the most studied cases of anti-TNF α mAbs, treatment with the human mAb adalimumab resulted in antibodies against the therapeutic that varied from <1% to up to 87% for different cohorts of patients, protocols, disease, and methods of measurement (39).

A likely mechanism for the immunogenicity of human mAbs involves the unique antibody sequences that confer antigen binding and specificity, but may appear foreign. Human therapeutic proteins can also break immune tolerance and aggregation can be a major determinant of antibody elicitation (34). Aggregation can result in repetitive structures that may not require T cell help (40). Protein immunogenicity may also affect efficacy through either the pharmacokinetic or neutralizing effects of the antibody responses that are dependent on a number of factors, including the affinity, specificity, and concentration of the induced antibodies (33). Because immunogenicity is an important factor in both safety and efficacy, significant efforts to predict and reduce immunogenicity of therapeutic proteins are ongoing (35–38).

Individual immune responses to therapeutic proteins vary widely. A key, and largely unanswered, question is what determines these variations. Despite extensive laboratory and clinical studies that were instrumental in delineating general concepts about critical factors involved in immunogenicity, it is impossible to predict the extent to which a novel therapeutic protein will be immunogenic in human patients. Little is known about the individual antibodies composing the polyclonal response to therapeutic proteins. The germline antibody repertoire at any given time could be a major determinant of individual differences, and so knowledge of large portions of antibodies generated by the human immune system, preferably the complete set, i.e., the antibodyome (6), could ultimately help to predict individual immune responses to therapeutic proteins.

In spite of the possibility for immunogenicity and other side effects, protein therapeutics are relatively safe due primarily to their high specificity. This is a fundamental advantage compared to small molecule drugs which on average are less specific and can bind nonspecifically to large number of molecules. However, in some cases, there are significant side effects, and safety concerns can lead to the withdrawal of therapeutic proteins from the market. The psoriasis drug efalizumab was withdrawn because of a potential risk of patients developing progressive multifocal leukoencephalopathy (PML), which is a rare, serious, progressive neurologic disease caused by the JC virus (JCV). More than 80% of the general population is infected with JCV. Why the virus becomes activated and causes disease only in minority of the treated patients is unknown, although typically PML occurs in people whose immune systems have been severely weakened. Thus, choosing the most appropriate animal model for toxicity testing is very important and species cross-reactivity should be included when identifying new candidate mAb therapeutics. If such a model does not exist, transgenic

animals expressing the human target and surrogate protein that is cross-reactive with the human homologous target in relevant animals can be used (41).

4.3. Efficacy

After safety, efficacy is the most important parameter considered by FDA for approval. Many therapeutic proteins are highly effective in vivo and have revolutionized treatment of diseases, e.g., insulin for diabetes, epoetin for anemia, and rituximab for non-Hodgkin lymphoma (32), to name a few. Alemtuzumab plays an important role in the therapy of hematological malignancies (42). Another example is trastuzumab as adjuvant systemic therapy for human epidermal growth factor receptor type 2 (HER2)-positive breast cancer (43). Results from six trials randomizing more than 14,000 women with HER2-positive early breast cancer to trastuzumab versus non-trastuzumab-based adjuvant chemotherapy demonstrate that the addition of trastuzumab reduces recurrence by approximately 50% and improves overall survival by 30% (44).

On average, the efficacy of therapeutic mAbs and some other therapeutic proteins is not high and there is substantial individual variability. One prominent example is trastuzumab (Herceptin) which has clearly revolutionized the treatment of HER2-positive patients; however, half of the patients still have non-responding tumors, and disease progression occurs within a year in the majority of cases (45). For patients with disease progression, combination with small molecules could be useful, e.g., the addition of a dual tyrosine kinase inhibitor of epidermal growth factor receptor (EGFR) and HER2 lapatinib to capecitabine was shown to provide superior efficacy for women with HER2-positive, advanced breast cancer progressing after treatment with anthracycline-, taxane-, and trastuzumab-based therapy (46). Current data do not support the use of trastuzumab for more than 1 year; the appropriate length of treatment, optimum timing, and administration schedule are not known (43). Like other therapeutic proteins trastuzumab does not appear to efficiently cross the blood–brain barrier, and it is unclear if the current practice of local therapy of the central nervous system and continued trastuzumab is optimal (45).

Anti-angiogenic therapies that target the vascular endothelial growth factor (VEGF), e.g., bevacizumab, and the VEGF receptor (VEGFR) are effective adjuncts for treatment of solid tumors, and are commonly administered in combination with cytotoxic chemotherapy. However, at least half of patients fail to respond to anti-angiogenic treatment of gliomas, and the response duration is modest and variable (47). The use of bevacizumab plus paclitaxel as a first-line treatment of patients with metastatic breast cancer doubled median progression-free survival (PFS; 11.8 months versus 5.9 months; hazard ratio = 0.60; $P < .001$) compared with paclitaxel alone; however, a statistically significant improvement in overall survival was not provided by the addition of bevacizumab, although a post hoc analysis demonstrated a significant increase in 1-year survival for the combination arm (48).

The anti-EGFR mAbs cetuximab and panitumumab, either as single agents or in combination with chemotherapy, have demonstrated clinical activity against metastatic colorectal cancer, but appear to benefit only select patients with predictive markers of efficacy, including EGFR overexpression, development of skin rash, and the absence of a K-ras mutation (49). In general, as single agents or in combination, therapeutic mAbs and other

proteins have produced only modest clinical responses in solid tumors (50). There are no mAbs approved for treatment of a number of tumors, e.g., prostate cancer. However, for prostate cancer, there are 30 candidates in the pipeline (16 vaccines and 14 antibodies), and one FDA-approved prostate cancer vaccine (Provenge); of these candidates, 19 are in phases II and III (9 vaccines and ten antibodies) and 8 are in phase I clinical trials.

The mechanisms underlying the relatively low efficacy of some therapeutic proteins and the high variability of responses to treatment are not well known, but are likely to involve multiple factors. Preexisting resistance or development of resistance is a fundamental problem for any therapeutic. Various mechanisms, including mutations, activation of multidrug transporters, and overexpression or activation of signaling proteins, are operating as exemplified for EGFR-targeted therapies (51). Another major problem is poor penetration into tissues, e.g., solid tumors. A related issue for full-size mAbs is poor or absent binding to regions on the surface of some molecules, i.e., existence of “steric barriers,” e.g., on the HIV envelope glycoprotein (Env) (22).

New approaches are being developed to increase efficacy of mAb and other therapeutic proteins, including enhanced effector functions, improved half-life, increased tumor and tissue accessibility, and greater stability; the methods used involve both protein- and glyco-engineering, and results to date are encouraging (52, 53). mAbs that do not engage the innate immune system's effector functions are being developed when binding is sufficient (54). Multi-targeted antibodies are being developed and tested in clinical trials, e.g., an antibody targeting HER2/neu and CD3 with preferential binding to activating Fc γ type I/III-receptors, resulting in the formation of tri-cell complexes among tumor cells, T cells, and accessory cells (55). Similar bispecific (targeting CD3 and epithelial cell adhesion molecule, EpCAM) trifunctional mAb, catumaxomab, was approved in the European Union for therapy of malignant ascites in 2009 (Table 2): the first bispecific mAb approved for clinical use. This antibody binds to cancer cells expressing EpCAM on their surface via one arm; to a T lymphocyte expressing CD3 via the other arm; and to an antigen-presenting cell like a macrophage, a natural killer cell, or a dendritic cell via the Fc. This initiates an immunological reaction leading to the removal of cancer cells from the abdominal cavity, thus reducing the tumor burden which is seen as the cause for ascites in cancer patients. Bispecific and multispecific mAbs and other therapeutic proteins are currently being developed to a number of targets.

A promising direction is the modulation of immune responses by mAbs targeting regulators of T cell immune responses. The cytotoxic T lymphocyte antigen 4 (CTLA-4) present on activated T cells is an inhibitory regulator of such responses. Human antibodies and Fc fusion proteins that abrogate the function of CTLA-4 have been tested in the clinic and found to have clinical activity against melanoma (56, 57). It appears that CTLA-4 blockade also enhanced the cancer-testis antigen NY-ESO-1-specific B cell and T cell immune responses in patients with durable objective clinical responses and stable disease suggesting immunotherapeutic designs that combine NY-ESO-1 vaccination with CTLA-4 blockade (57). Ipilimumab which targets CTLA-4 was approved by the US FDA in 2011 for therapy of metastatic melanoma (Table 2). Therapeutic mAbs that mimic the natural ligand, e.g., the

tumor necrosis factor-related apoptosis inducing ligand (TRAIL), have also been developed (58, 59).

Currently, second- and third-generation mAbs against already validated targets, e.g., HER2, CD20, and TNF α , are in clinical studies or already approved. Various approaches have been used to discover novel, relevant targets, but progress has been slow. Modifications of the standard panning procedures have been reported, including enhanced selection of cross-reactive antibodies by sequential antigen panning (60) and competitive antigen panning for focused selection of antibodies targeting a specific protein domain or subunit (61, 62). To ensure better tissue penetration and hidden epitope access, a variety of small engineered antibody domains (about tenfold smaller than IgG) are being developed (19, 20). Knowledge of antibodyomes could be used for generation of semisynthetic libraries for selection of high-affinity binders of small size and minimal immunogenicity (6).

A major lesson from the current state of antibody-based therapeutics is that gradual improvement in the properties of existing therapeutic proteins and identification of novel proteins and targets are likely to continue in the foreseeable future. A fundamental challenge has been to increase dramatically the efficacy of therapeutic antibodies and to apply them to many more diseases. Other major challenges are the development of effective personalized antibody-based therapeutics, and prediction of toxicity or potentially low efficacy in vivo.

4.4. Quality

Quality is a very important parameter for approval of any drug by FDA. A specific fundamental feature that distinguishes mAb and other biologics from small molecule drugs is their heterogeneity. Heterogeneity of mAbs is due to modifications, such as incomplete disulfide bond formation, glycosylation, N-terminal pyroglutamine cyclization, C-terminal lysine processing, deamidation, isomerization, oxidation, amidation of the C-terminal amino acid, and modification of the N-terminal amino acids by maleuric acid, as well as noncovalent associations with other molecules, conformational diversity, and aggregation (63). Tens of thousands of variants with the same sequence may coexist.

Development of high-quality protein therapeutics with minimal heterogeneity and contamination is essential for their safety and approval by FDA. Process development for production of therapeutic proteins is a very complex operation involving recombinant DNA technologies, verification of a strong expression system, gene amplification, characterization of a stable host cell expression system, optimization and design of the mammalian cell culture fermentation system, and development of an efficient recovery process resulting in high yields and product quality (64). Titers in the range of 5–10 g/L or even higher, cell densities of more than 20 million cells/ml, and specific productivity of over 20 pg/cell/day (even up to 100 pg/cell per day) have been achieved (65).

Genetic delivery of therapeutic proteins by in vivo production offers a new direction to increase quality and reduce cost; three approaches can be used for the stable long-term expression and secretion of therapeutic proteins in vivo: (1) direct in vivo administration of integrating vectors carrying the gene, (2) grafting of ex vivo genetically modified autologous cells, and (3) implantation of an encapsulated antibody producing heterologous or

autologous cells. Another promising direction is the prospects for using molecular farming methods to create relatively low-cost therapeutic proteins in plants, e.g., in genetically engineered tobacco leaves.

5. Biosimilar and Biobetter Therapeutic Proteins

A major direction of current activity is to develop therapeutic proteins that are similar but cheaper than the currently existing or are better in terms of efficacy and safety. By 2015, biologics worth \$60 billion in annual sales will lose patent protection, bolstering hopes for the rapid growth of the biosimilars as generics companies elbow their way into a big new market. Rituxan/MabThera, Remicade, and Enbrel are on the top of the list for biosimilars. Sandoz, e.g., which is leading the pack of generic companies angling to get into the market, expects to see biosimilar revenue jump from \$250 million in 2011 to \$20 billion by 2020. Over the next 5 years, the market for biosimilars will increase to \$10 billion, but only a handful of big pharmaceutical companies and world-class R&D facilities will be able to take part. And that means that most small- and medium-size drug developers will never have a chance of getting into the new market for follow-on biologics.

The niche for most small biotech companies is taking a preclinical- or very-early-stage candidate to proof of concept, at which point they can make sale to bigger companies. With biosimilars, the developer will start with proof of concept data and then ramp up the most expensive stage of clinical development, with the added charge of running a likely comparison study to the marketed therapeutic. That will not be cheap. It could take 8 years to run a biosimilar program with development costs sliding from \$100 million to \$150 million. With that much time and money at stake, most biotech companies may never be competitive.

6. Conclusions

The rapid progress made in the last few decades toward the development of potent therapeutic proteins raises a number of questions for the future directions of this field. A key question is whether there are any indications of a paradigm change that could lead to radically different therapeutics as occurred two to three decades ago and which resulted in an explosion of protein therapeutics approved for clinical use during the last decades. If history provides an answer and such a paradigm shift occurs, it will probably take decades before we witness the fruition of such a shift in terms of new licensed protein therapeutics. Meanwhile, gradual improvements in the characteristics of existing protein therapeutics, discovery of novel protein-based drugs and novel targets, combining therapeutics, conjugating them with drugs, nanoparticles, and other reagents using integrative approaches based on cell biology, bioengineering and genetic profiling, as well as predictive tools to narrow down which candidate molecules could be successfully developed as therapeutics, and developing novel protein-based scaffolds with superior properties to those already in use will be major areas of research and development in the coming decades. A decade from now, it is likely that we will see many protein-based therapeutics based on different scaffolds approved for clinical use and hundreds more in preclinical and clinical development.

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Table 1

The 30 top-selling therapeutic proteins in 2010 (in bln US\$) (modified from LaMerie Business Intelligence, Barcelona)

# (09)	Name	Target/mechanism	Type	Company	Indication	Sales
1 (1)	Etanercept	TNF α	Fc fusion TNFR2 ECD	Amgen Wyeth	Immune diseases	7,287
2 (3)	Bevacizumab	VEGF	Humanized IgG	Genentech Roche Chugai	Cancer	6,973
3 (4)	Rituximab	CD20	Chimeric IgG	Genentech Biogen-IDEC Roche	Cancer	6,859
4 (5)	Adalimumab	TNF α	Human IgG	Abbott Eisai	Immune diseases	6,548
5 (2)	Infliximab	TNF α	Chimeric IgG	Centocor (J&J) Schering-Plough Mitsubishi Tanabe	Immune diseases	6,520
6 (7)	Trastuzumab	Her2	Humanized IgG	Genentech Chugai Roche	Cancer	5,859
7 (8)	Insulin glargine	Insulin receptor	Modified insulin	Sanofi-Aventis	Diabetes	4,834
8 (6)	Epoetin alfa	EPO-R	Human EPO	Amgen Ortho Biotech Kyowa Hakko Kirin	Anemia	4,590
9 (9)	Pegfilgrastim	G-CSF receptor	PEGhuman G-CSF	Amgen	Neutropenia	3,558
10 (11)	Ranibizumab	VEGF	Humanized Fab	Genentech Novartis	AMD	3,106
11 (10)	Darbepoetin alfa	EPO-R	Modified human EPO	Amgen Kyowa Hakko Kirin	Anemia	2,995
12 (12)	Interferon beta-1a (Avonex)	Interferon beta receptor	Human interferon beta-1a	Biogen Idec	Multiple sclerosis	2,518
13 (13)	Interferon beta-1a (Rebif)	Interferon beta receptor	Human interferon beta-1a	Merck Serono	Multiple sclerosis	2,297
14 (17)	Insulin aspart	Insulin receptor	Modified insulin	Novo Nordisk	Diabetes	2,198
15 (14)	Rhu insulin	Insulin receptor	Modified insulin	Novo Nordisk	Diabetes	2,185
16 (15)	Octocog alfa	Factor VIII replacement	Factor VIII	Baxter Healthcare	Hemophilia A	2,095
17 (16)	Insulin lispro	Insulin receptor	Modified insulin	Eli Lilly	Diabetes	2,054
18 (19)	Cetuximab	EGF-R	Chimeric IgG	Eli Lilly BMS Merck Serono	Cancer	1,791
19 (20)	Peginterferon alfa-2a	Interferon alfa receptor	PEGhuman protein	Roche	Hepatitis C	1,775
20 (18)	Interferon beta-1b	Interferon beta receptor	Human protein	Berlex Bayer Schering	Multiple sclerosis	1,661
21 (23)	Eptacog alfa	Initiate coagulation	Human factor VIIa	Novo Nordisk	Hemophilia	1,483
22 (25)	Insulin aspart	Insulin receptor	Modified insulin	Eli Lilly	Diabetes	1,445
23 (-)	OnabotulinumtoxinA	SNAP-25 cleavage	Botulinum toxin type A	Allergan GSK	Medical and esthetic	1,414
24	Epoetin beta	EPO-R	Human EPO	Roche	Anemia	1,387
25	Rec antihemophilic factor	F VIII substitution	Human protein	Bayer Schering	Hemophilia A	1,383
26	Filgrastin	G-CSF receptor	Human protein	Amgen	Neutropenia	1,286

# (09)	Name	Target/mechanism	Type	Company	Indication	Sales
27	Insulin detemir	Insulin receptor	Modified insulin	Novo Nordisk	Diabetes	1.271
28	Natalizumab	$\alpha_4\beta_1/7$ integrin	Humanized IgG	Biogen Idec Elan	Multiple sclerosis	1.230
29	Insulin (humulin)	Insulin receptor	Human insulin	Eli Lilly	Diabetes	1.089
30	Palivizumab	RSV	Humanized IgG	MedImmune	RSV	1.038

The numbers in parentheses are for year 2009

Currencies as of March 2, 2011: 1 €= 1.37726 US\$; 1 CHF = 1.07917 US\$; 1 Yen = 0.0121955 US\$; 1 DKK=0.184739 US\$; 1 SEK=0.157766 US\$

Therapeutic monoclonal antibodies approved or in review in the European Union or the USA

Table 2

Name	Trade name	Type	Indication first approved	First EU (US) approval year
Murmonab-CD3	Orthoclone Okt3	Anti-CD3; Murine IgG2a	Reversal of kidney transplant rejection	1986 ^a (1986#2010@)
Abciximab	Reopro	Anti-GPIIb/IIIa; Chimeric IgG1 Fab	Prevention of blood clots in angioplasty	1995 ^a (1994)
Rituximab	MabThera, Rituxan	Anti-CD20; Chimeric IgG1	Non-Hodgkin's lymphoma	1998 (1997)
Basiliximab	Simulect	Anti-IL2R; Chimeric IgG1	Prevention of kidney transplant rejection	1998 (1998)
Daclizumab	Zenapax	Anti-IL2R; Humanized IgG1	Prevention of kidney transplant rejection	1999 (1997); #2009
Palivizumab	Synagis	Anti-RSV; Humanized IgG1	Prevention of respiratory syncytial virus infection	1999 (1998)
Infliximab	Remicade	Anti-TNF; Chimeric IgG1	Crohn's disease	1999 (1998)
Trastuzumab	Herceptin	Anti-HER2; Humanized IgG1	Breast cancer	2000 (1998)
Gemtuzumab ozogamicin	Mylotarg	Anti-CD33; Humanized IgG4	Acute myeloid leukemia	NA (2000#2010)
Alemtuzumab	MabCampath, Campath-1H	Anti-CD52; Humanized IgG1	Chronic myeloid leukemia	2001 (2001)
Adalimumab	Humira	Anti-TNF; Human IgG1	Rheumatoid arthritis	2003 (2002)
Tositumomab + ¹³¹ I-Tositumomab	Bexxar	Anti-CD20; Murine IgG2a	Non-Hodgkin lymphoma	NA (2003)
Efalizumab	Raptiva	Anti-CD11a; humanized IgG1	Psoriasis	2004 (2003); #2009
Cetuximab	Erbix	Anti-EGFR; chimeric IgG1	Colorectal cancer	2004 (2004)
Ibritumomab tiuxetan	Zevalin	Anti-CD20; murine IgG1	Non-Hodgkin's lymphoma	2004 (2002)
Omalizumab	Xolair	Anti-IgE; humanized IgG1	Asthma	2005 (2003)
Bevacizumab	Avastin	Anti-VEGF; humanized IgG1	Colorectal cancer	2005 (2004)
Natalizumab	Tysabri	Anti-a4 integrin; humanized IgG4	Multiple sclerosis	2006 (2004)
Ranibizumab	Lucentis	Anti-VEGF; humanized IgG1 Fab	Macular degeneration	2007 (2006)
Panitumumab	Vectibix	Anti-EGFR; human IgG2	Colorectal cancer	2007 (2006)
Eculizumab	Soliris	Anti-C5; humanized IgG2/4	Paroxysmal nocturnal hemoglobinuria	2007 (2007)
Certolizumab pegol	Cimzia	Anti-TNF; humanized Fab, pegylated	Crohn disease	2009 (2008)
Golimimumab	Simponi	Anti-TNF; human IgG1	Rheumatoid and psoriatic arthritis, ankylosing spondylitis	2009 (2009)
Canakinumab	Ilaris	Anti-IL1b; human IgG1	Muckle-Wells syndrome	2009 (2009)
Catumaxomab	Removab	Anti-EPCAM/CD3;rat/mouse bispecific mAb	Malignant ascites	2009 (NA)
Ustekinumab	Stelara	Anti-IL12/23; human IgG1	Psoriasis	2009 (2009)
Tocilizumab	RoActemra, Actemra	Anti-IL6R; humanized IgG1	Rheumatoid arthritis	2009 (2010)

Name	Trade name	Type	Indication first approved	First EU (US) approval year
Ofatumumab	Arzerra	Anti-CD20; human IgG1	Chronic lymphocytic leukemia	2010 (2009)
Denosumab	Prolia	Anti-RANK-L; human IgG2	Bone loss	2010 (2010)
Belimumab	Benlysta	Anti-BLyS; human IgG1	Systemic lupus erythematosus	(2011)
Raxibacumab	(Pending)	Anti- <i>B. anthracis</i> PA; human IgG1	Anthrax infection	NA (in review)
Ipilimumab	Yervoy	Anti-CTLA-4; human IgG1	Metastatic melanoma	(2011)
Brentuximab vedotin	(Pending)	Anti-CD30; chimeric IgG1; immunoconjugate	Hodgkin lymphoma, systemic ALCL	NA (application submitted)

Information current as of July 2011. Updated and modified from Janice M. Reichert, Editor-in-Chief, mAbs

^aCountry-specific approval; approved under concertation procedure; #Voluntarily withdrawn from the market. @Supplies are exhausted in 2010. *BLyS* B lymphocyte stimulator, C5 complement 5, *CD* cluster of differentiation, *CTLA-4* cytotoxic T lymphocyte antigen 4, *EGFR* epidermal growth factor receptor, *EPCAM* epithelial cell adhesion molecule, *GP* glycoprotein, *IL* interleukin, *NA* not approved, *PA* protective antigen, *RANK-L* receptor activator of NFκB ligand, *RSV* respiratory syncytial virus, *TNF* tumor necrosis factor, *VEGF* vascular endothelial growth factor. Not included here are polyclonal antibodies against infectious diseases and toxins

Table 3

List of Fc fusion proteins, the year denotes date of approval

-
1. Etanercept (TNFR2 ECD, 1998)
 2. Alefacept (LFA3 ECD, 2003)
 3. Abatacept (CTLA4 ECD, 2005)
 4. Rilonacept (IL-1RI/IL-1RacP ECD, 2008)
 5. Romiplostim (41aa thrombopoietin (TPO) analogue peptide, 2008)
 6. Belatacept (CTLA4 ECD, 2011)
-

ECD extracellular domain

Table 4

List of genuine noncovalent binders other than mAbs, Fc fusion proteins, and polyclonal immunoglobulins approved for clinical use

1. Insulin (blood glucose regulator)
2. Pramlintide acetate (glucose control)
3. Growth hormone GH (growth failure)
4. Pegvisoman (growth hormone receptor antagonist)
5. Mecasermin (IGF1, growth failure)
6. Factor VIII (coagulation factor)
7. Factor IX (coagulation factor)
8. Protein C concentrate (anti-coagulation)
9. α 1-proteinase inhibitor (anti-trypsin inhibitor)
10. Erythropoietin (stimulates erythropoiesis)
11. Filgrastim (granulocyte colony-stimulating factor, G-CSF; stimulates neutrophil proliferation)
12. Sargramostim ^{36, 37} (granulocytemacrophage colony-stimulating factor, GM-CSF)
13. Oprelvekin (interleukin11, IL11)
14. Human follicle-stimulating hormone (FSH)
15. Human chorionic gonadotropin (HCG)
16. Lutropin- α (human luteinizing hormone)
17. Interleukin 2 (IL2)
18. Denileukin diftitox (fusion of IL2 and Diphtheria toxin)
19. Interferon alfacon 1 (consensus interferon)
20. Interferon- α 2a (IFN α 2a)
21. Interferon- α 2b (IFN α 2b)
22. Interferon- α n3 (IFN α n3)
23. Interferon- β 1a (rIFN- β)
24. Interferon- β 1b (rIFN- β)
25. Interferon- γ 1b (IFN γ)
26. Salmon calcitonin (32-amino acid linear polypeptide hormone)
27. Teriparatide (part of human parathyroid hormone 1–34 residues)
28. Exenatide (Incretin mimetic with actions similar to glucagon-like peptide 1)
29. Octreotide (octapeptide that mimics natural somatostatin)
30. Dibotermine- α (recombinant human bone morphogenic protein 2)
31. Recombinant human bone morphogenic protein 7
32. Histrelin acetate (gonadotropin-releasing hormone; GnRH)
33. Palifermin (keratinocyte growth factor, KGF)
34. Becaplermin (platelet-derived growth factor, PDGF)
35. Nesiritide (recombinant human B-type natriuretic peptide)
36. Lepirudin (recombinant variant of hirudin, another variant is Bivalirudin)
37. Anakinra (interleukin 1 (IL1) receptor antagonist)
38. Enfuvirtide (an HIV-1 gp41-derived peptide)

Information for the protein and/or abbreviations used is provided in parentheses. Modified and updated from (8)

Table 5

List of genuine therapeutic proteins affecting covalent bonds—enzymes and antithrombin III—approved for clinical use

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1. β -Glucocerebrosidase (hydrolyzes to glucose and ceramide)
 2. α -Glucosidase (degrades glycogen)
 3. Laronidase (digests glycosaminoglycans within lysosomes)
 4. Idursulfase (cleaves O-sulfate preventing GAGs accumulation)
 5. Galsulfase (cleaves terminal sulphate from GAGs)
 6. Agalsidase- β (human α -galactosidase A, hydrolyzes glycosphingolipids)
 7. Lactase (digest lactose)
 8. Pancreatic enzymes (lipase, amylase, protease; digest food)
 9. Adenosine deaminase (metabolizes adenosine)
 10. Tissue plasminogen activator (tPA, serine protease involved in the breakdown of blood clots)
 11. Factor VIIa (serine protease, causes blood to clot)
 12. Drotrecogin- α (serine protease, human activated protein C)
 13. Trypsin (serine protease, hydrolyzes proteins)
 14. Botulinum toxin type A (protease, inactivates SNAP-25 which is involved in synaptic vesicle fusion)
 15. Botulinum toxin type B (protease that inactivates SNAP-25 which is involved in synaptic vesicle fusion)
 16. Collagenase (endopeptidase, digest native collagen)
 17. Human deoxyribonuclease I (endonuclease, DNase I, cleaves DNA)
 18. Hyaluronidase (hydrolyzes hyaluronan)
 19. Papain (cysteine protease, hydrolyzes proteins)
 20. L-Asparaginase (catalyzes the conversion of L-asparagine to aspartic acid and ammonia)
 21. Rasburicase (urate oxidase, catalyzes the conversion of uric acid to allantoin)
 22. Streptokinase (Anistreplase is anisoylated plasminogen streptokinase activator complex (APSAC))
 23. Antithrombin III (serine protease inhibitor)
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Information for the protein and/or abbreviations used is provided in parentheses. Modified and updated from (8)