Overview of Biopharmaceuticals and Comparison with Small-molecule Drug Development

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INTRODUCTION

Therapeutic proteins have been an important component of medical practice since the late nineteenth century, when the protective properties of passive immunization were discovered in blood transferred from pathogen-infected animals [1,2]. This important discovery was quickly followed by early twentieth century success with pancreatic extracts in the treatment of diabetes mellitus [3]. Recombinant DNA technology enabled the mass production of proteins and antibodies using living cells (bacterial, yeast, plant, insect, or mammalian) using well-defined bioprocess methods. The resulting products have a defined specificity and uniformity, which is a vast improvement over previous methods of extraction and purification of proteins from human or animal blood and tissues. Recombinant DNA-derived medicinal products are often interchangeably referred to as “biopharmaceuticals,” “biotherapeutics,” “biologicals,” or “biologics.”

This chapter introduces the various classes of therapeutics that are produced using recombinant DNA technology, and provides background on the history and evolution of therapeutic hormones, enzymes, cytokines, and monoclonal antibodies from an early understanding of their value in the treatment of disease to present day production of genetically engineered human proteins and novel constructs designed to improve uniformity, safety, efficacy, or duration of effect. The introduction of these products to the medical armamentarium heralded the beginning of the biotechnology industry and revolutionized medicine.

In order to bring these new medicines to patients, some specific considerations and different approaches compared to those previously established for small-molecule drugs were needed

http://dx.doi.org/10.1016/B978-0-12-394810-6.00001-0 © 2013 Elsevier Inc. All rights reserved.
to characterize the safety profile of biopharmaceuticals. A comparative review highlighting similarities and differences in the development of biopharmaceuticals and small-molecule drugs is included in this chapter.

HISTORY AND EVOLUTION OF BIOPHARMACEUTICALS

The First Protein Therapeutics

In the 1920s and 1930s, prior to the advent of prophylactic vaccines, “serum therapy,” derived from pathogen-infected animals, was employed to treat a variety of infectious diseases including diphtheria, scarlet fever, pneumococcal pneumonia, and meningococcal meningitis [4,5]. Despite relative success in the management of bacterial infections, systemic administration of a heterologous (non-human), mixture of immunoglobulins (Igs) resulted in high risk to patients for immunological toxicities such as allergic or anaphylactoid reactions. Improvements in sanitation and hygiene had a positive impact on both primary infection and contagion, and the discovery and development of antibiotics in the 1930s and 1940s provided a highly effective treatment alternative, which quickly became the standard of care for bacterial infections. As a consequence, the use of animal sera for passive immunization was reserved for toxin-mediated afflictions due to diphtheria, tetanus, botulism, and venomous bites [4–6].

Immunoglobulin preparations derived from human placenta and plasma have been in clinical use since the early to mid-1940s when gamma globulin injections were used for prevention or treatment of viral diseases. Intravenous immunoglobulin (IVIG) infusion continues to be a mainstay of treatment for antibody deficiency disorders and autoimmune and inflammatory conditions such as idiopathic thrombocytopenic purpura and Kawasaki syndrome [7]. In addition, hyperimmune IgG preparations (HIG) purified from the plasma of human donors that have been exposed to viruses such as respiratory syncytial virus (RSV), cytomegalovirus (CMV), or human immunodeficiency virus (HIV) continue to provide therapeutic or prophylactic benefit to vulnerable populations [8–11].

Early therapeutic proteins in clinical use were likewise derived initially from animal, and subsequently from human sources. The identification and purification of insulin from bovine pancreas in 1922 provided glucose control for diabetes patients who had no real treatment options [3]. Clotting factor VIII for hemophilia was initially derived from human plasma, β-glucocerebrosidase for Gaucher’s disease was initially purified from human placenta [12], and human growth hormone was derived from the pituitary of human cadavers [13]. Each of these products would later be replaced by homogeneous and well-characterized protein therapeutics produced through recombinant DNA technology.

Biopharmaceuticals Produced by Recombinant DNA Technology

In 1978, human insulin was produced through genetic engineering [14,15], and in 1982 it became the first biotechnology product to receive US Food and Drug Administration (FDA) approval [16]. The cloning and expression of human insulin ushered in the age of biotechnology and this achievement was rapidly followed by the cloning and expression of human
growth hormone [17], leading to US FDA approval in 1985, followed by approval of interferon alphas 2a and 2b in 1986 [16]. The production of large quantities of a single human protein improved patient access to life-saving treatment and reduced the risk of pathogen transmission, or an immune reaction to other animal or human proteins that were present in the product. The tragic consequences of unwitting hepatitis C and HIV transmission to hemophiliacs treated with plasma-derived clotting products in the 1980s lent urgency to the development of a recombinant factor VIII [18,19], as well as the development of screening tools for the blood supply [20].

Alongside gene identification, cloning, and protein expression, Köhler and Milstein’s [21] development of the technology to produce antibodies against a defined target stands as a watershed moment in biotechnology. The fusion of long-lived murine myeloma cells to murine spleen cells from an immunized donor to form a hybridoma capable of secreting antigen-specific antibodies enabled production of monoclonal antibodies as targeted therapeutics for a wide variety of diseases.

Technical developments in the production of antibody therapeutics are reflected in the chronology of marketing approvals. In 1986, muromonab-CD3 (OKT3®) was approved for use in acute transplant rejection. OKT3® is a wholly murine monoclonal antibody that was purified from a hybridoma generated via the fusion of a murine myeloma cell and a B cell from mice immunized with human CD3 [22,23]. To create the next generation of monoclonal antibodies, genes encoding the variable region of antibodies produced by murine hybridoma cell lines were ligated to the genes encoding the constant region of human IgG and transfected into murine myeloma [24,25], and later into immortalized mammalian cells [26–28] to produce chimeric antibodies with a defined specificity. Abciximab (Reopro®) is an antibody fragment (Fab) composed of the binding region only, eliminating the Fc portion, and was the first chimeric biotherapeutic to be approved for human use (1994), followed by the chimeric anti-CD20 antibody rituximab (Rituxan®) in 1997 [16].

Humanized monoclonal antibodies (mAbs) are produced by transplanting only the rodent residues required for antigen binding onto a human IgG framework. Daclizumab (Zenapax®) was the first humanized mAb to be approved for human use in 1997, followed by palivizumab (Synagis®) and trastuzumab (Herceptin®) in 1998 [16]. Fully human antibodies can be produced by phage display, where an antigen of interest is screened against a library of diverse human immunoglobulin variable region segments [29,30]. This technology was used to produce adalimumab (Humira®), the first fully human mAb granted marketing approval by the US FDA [31].

Following on the success of recombinant protein replacement therapies, recombinant proteins expanded into cancer with the 1986 marketing approval of recombinant interferon alphas 2a and 2b (Roferon A®, Intron A®, respectively), for the treatment of hairy cell leukemia, a subtype of chronic lymphoid leukemia that affected just 2% of all US leukemia patients at that time [32]. Because of the higher costs of producing biopharmaceutical products relative to small-molecule pharmaceuticals and because proteins require parenteral administration, biopharmaceuticals were niche products in the early years, indicated as replacement therapy, acute treatment for life-threatening indications, or for difficult-to-treat disease areas refractory to the standard of care such as cancer [16,30]. As the underlying mechanisms of disease were elucidated and positive patient outcomes with acceptable benefit/risk profiles emerged with biopharmaceuticals, their use was expanded
into chronic diseases, including autoimmune disorders such as asthma, multiple sclerosis, and rheumatoid arthritis [16,31,33].

Recombinant DNA technology made it possible to produce therapeutic human proteins at a large scale with greater purity, homogeneity, stability, and predictable potency than had been available from protein products extracted from animal and human blood and tissues. The state of the art has evolved from one of reduction—purifying a single protein from large quantities of complex, heterogeneous human or animal protein mixture—to a model of controlled expansion: cloning a gene encoding a protein of interest into a prokaryotic or eukaryotic cell and selectively expressing large quantities of a single human protein. This has the advantage of eliminating the need for sources of human plasma (with attendant concerns over pathogenic agents), while improving protein yields and product uniformity.

The Emergence of Novel Constructs

Technological advances in protein and antibody engineering have provided the tools to design biopharmaceuticals with attributes to improve systemic exposure, efficacy, product stability, and safety. For example, site-directed mutagenesis was used to engineer recombinant hemoglobin with the oxygen affinity and stable tetrameric structure necessary for efficient oxygen dissociation to tissues without the renal damage caused by smaller constructs [34,35]. Human insulin has been similarly engineered to improve half-life [36,37] and to reduce aggregation for improved onset of activity [38]. Conjugation of therapeutic proteins to inert polymers such as polyethylene glycol (PEG) to prolong plasma half-life, reduce frequency of administration, and enhance efficacy has provided PEGylated treatment options such as interferon alpha-2a (Pegasys®), interferon alpha-2b (PegIntron A®, ViraferonPeg®), and GM-CSF (Neulasta®). More recent forms of protein engineering include the creation of fusion proteins such as Ontak® (denileukin diftitox; recombinant IL-2+diphtheria toxin), Enbrel® (etanercept; recombinant TNF receptor+IgG Fc), and Amevive® (alefacept; LFA-3+IgG Fc) [31].

Modification of the glycosylation sites of proteins produced in mammalian cells can confer distinct properties. Hyperglycosylation of erythropoietin to produce Aranesp® (darbepoetin alfa) improved pharmacokinetic properties [39], while afucosylation of mAbs has been shown to enhance binding to FcγRIII and improve effector functions such as antibody-dependent cellular cytotoxicity (ADCC) [40,41]. Other structural alterations to IgGs include amino acid substitutions to the complement component C1q-binding sites to increase complement-dependent cytotoxicity (CDC) activity [42], FcRn mutations to improve plasma half-life through antibody recycling and prevention of lysosomal degradation [43], and modification of hinge regions to positively or negatively modulate both ADCC and CDC effector functions [44].

As of 2010, over 200 biopharmaceuticals have been approved for human use, with clinical indications spanning cancer, autoimmune disorders, metabolic imbalances, and infectious disease [31,45,46]. In the 30 years since recombinant human insulin was first expressed in the laboratory, recombinant DNA technology has made important contributions to medical science and forged new directions in regulatory decision making, with a new approach to characterizing the toxicity of new molecular entities (NMEs). Advances in genetic engineering
technology and a greater understanding of disease biology provide an opportunity to draft a prospective blueprint of an NME with a number of desired biopharmaceutical attributes with effects on primary pharmacology, pharmacokinetic properties, and manufacturing ease.

**DEVELOPMENT OF DIVERSE BIOPHARMACEUTICAL MODALITIES**

**Enzyme Replacement Therapy**

Lysosomal storage disorders are characterized by the abnormal accumulation of metabolic substrates in the lysosomes of many types of cells and tissues of the body. Substrate accumulation results from a deficiency of specific lysosomal enzymes required for the degradation of macromolecules. Examples of lysosomal storage disorders include Gaucher’s disease (sphingolipidosis), mucopolysaccharidosis (MPS) types I, II, and IV, Pompe disease (glycogen storage disease type II), and Fabry disease (sphingolipidosis). The aim of enzyme replacement therapy (ERT) is to replace deficient or dysfunctional enzymes to facilitate the proper processing of cellular macromolecules and prevent their buildup inside cells and the resulting pathophysiological consequences. Examples of approved ERTs are shown in Table 1.1. Early clinical trials demonstrated proof of concept for ERTs via administration of the deficient normal human enzyme, however the notable limitation of this approach was poor pharmacokinetics; the highly purified human enzymes were cleared from the circulation rapidly ($T_{1/2}$ of 10–20 min) [47]. The glycosylation of endogenously synthesized lysosomal enzymes enables their targeting to the lysosome via the mannose-6-phosphate receptor [48,49] and protein engineering efforts have focused on designing analogously glycosylated biotechnology-derived enzyme replacement therapies. Because of this requirement for specific glycosylation, the

<table>
<thead>
<tr>
<th>Initial approval (year/country)</th>
<th>Brand name</th>
<th>Active compound</th>
<th>Initial indication</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1994 (US)</td>
<td>Cerezyme</td>
<td>Imiglucerase (recombinant human β-glucocerebrosidase)</td>
<td>Gaucher’s disease</td>
<td>Genzyme</td>
</tr>
<tr>
<td>2003 (US)</td>
<td>Aldurazyme</td>
<td>Laronidase (recombinant α-1-iduronidase)</td>
<td>Mucopolysaccharidosis I</td>
<td>Genzyme/Biomarin Pharmaceutical</td>
</tr>
<tr>
<td>2006 (US)</td>
<td>Elaprase</td>
<td>Idursulfase (recombinant human iduronate-2-sulfatase)</td>
<td>Mucopolysaccharidosis II (Hunter’s syndrome)</td>
<td>Shire</td>
</tr>
<tr>
<td>2010 (US)</td>
<td>Myozyme</td>
<td>Alglucosidase α (recombinant human acid-α-glucosidase)</td>
<td>Pompe disease (glycogen storage disease type II)</td>
<td>Genzyme</td>
</tr>
<tr>
<td>2010 (US)</td>
<td>VPRIV</td>
<td>Velaglucerase α (human glucocerebrosidase)</td>
<td>Gaucher’s disease</td>
<td>Shire</td>
</tr>
</tbody>
</table>
recombinant therapeutic enzymes, in contrast to other recombinant biological drugs, must be manufactured using mammalian cells (e.g. Chinese hamster ovary (CHO) cells), because the prokaryotic cell systems commonly used in biotechnology manufacturing (e.g. *E. coli*) are unable to execute the particular post-translational modifications needed for lysosomal stability and activity.

A notable limitation of present therapies is that exogenously administered enzymes, by virtue of their macromolecular structure and size, do not have access to the brain which is protected by the blood–brain barrier. Most of the lysosomal storage disorders have neurologic sequelae, and the inability of the biologic therapies to access brain tissue is a significant limitation to their overall effectiveness [50]. Intrathecal administration is one possible solution to address this issue; although technically challenging, short-term administration via this route may be a promising option for patients with lysosomal storage disorders affecting the brain. However, long-term administration via the intrathecal route is not reasonable due to inherent concerns about the safety and clinical practicality of repeated lumbar puncture. Next-generation therapies will likely attempt to address this limitation by modifications of the protein structure and/or delivery methodology.

Because the number of patients that are affected by lysosomal storage disorders is very small, enzyme replacement therapies have typically been pursued under the FDA’s Orphan Drug Act. This legislation recognizes that the high cost of development may limit treatments for small patient populations. The Act provides assistance to promote the development of treatments, including enhanced patent protection and financial incentives. The nonclinical development strategy for an orphan drug, however, should not differ dramatically for a more conventional indication.

**Engineering of Therapeutic Proteins**

Over time, biotechnology-derived products have evolved from simple re-creations of the native, endogenous protein and have become increasingly engineered and complex. Safety-related events largely prompted the development of a biotechnology-derived therapeutic; examples include insulin, which was originally derived from bovine pancreas, and human growth hormone, which was extracted from human pituitaries. The original insulin preparations were relatively crude by current standards. They contained extraneous proteins such as proinsulin, glucagon and somatostatin, as well as modified forms of insulin such as desamidoinsulin [51]. While the protease contaminant was a concern because of the potential for hydrolysis of insulin, which could consequently decrease therapeutic efficacy, the presence of bovine proinsulin was a safety-related concern because of its immunogenicity in humans. Transmission of Creutzfeldt–Jakob disease (CJD) has been observed following administration of pituitary-derived human growth hormone preparations and is responsible for hundreds of deaths worldwide in recipients of these preparations; the advent of recombinant human growth hormone completely prevented this unfortunate side effect [52]. Since the development of insulin, many other hormones, as well as growth factors and cytokines, have been developed using biotechnology capabilities (Table 1.2).
I. DEVELOPMENT OF BIOPHARMACEUTICALS DEFINED AS NOVEL BIOLOGICS

**Structural modifications**

Protein engineering efforts have focused on improving efficacy and pharmacokinetic profiles by modifying the structural attributes of recombinant proteins. For example, in the case of insulin, it was recognized that upon storage of a drug product composed of individual native insulin molecules, dimeric and hexameric insulin structures were formed. In vivo, these higher order species had a longer residence time at the site of injection (either subcutaneous or intramuscular), which resulted in a slower onset of the therapeutic effect. To mitigate this challenge, alterations of the amino acid sequence in the regions of the protein associated with...
the propensity for self-association diminished aggregation and thus generated faster acting insulins (i.e. Humalog®/Liprolog®) [51].

Insulin has also been the subject of protein engineering efforts to prolong the duration of action in vivo. Levemir® (approved in the EU in 2004) was modified to enable reversible binding to albumin both at the site of injection and subsequently in the plasma, which permits prolonged release of insulin and increased duration of action of the drug (fivemfold improvement in half-life) [53].

**PEGylation**

Proteins can be rapidly degraded in vivo by proteases and prolonging time in circulation can improve therapeutic utility. In addition, the therapeutic benefit of proteins may be compromised by the formation of anti-drug antibodies (ADAs) (immunogenicity) that can hasten the elimination of the protein from circulation or, in some cases, elicit adverse effects [54–56]. Efforts to improve pharmacokinetics and mitigate the immunogenicity of proteins have included the use of polyethylene glycol (PEG) conjugation. PEG polymers are composed of repeating units of ethylene glycol arranged linearly or in branched configurations. PEG is attached to the protein of interest, either covalently or by a hydrolysable linkage [57], and this modification effectively increases the time that the protein remains in circulation by decreasing the rate of renal clearance and providing protection from proteolytic degradation [58,59]. By a similar mechanism, namely steric hindrance or allosteric modification, PEGylation of proteins can minimize exposure of antigenic determinants, thereby mitigating the immunogenicity of the therapeutic [60]. Examples of approved PEGylated biopharmaceuticals are shown in Table 1.3.

<table>
<thead>
<tr>
<th>Initial approval (year/country)</th>
<th>Brand name</th>
<th>Active compound</th>
<th>Initial indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1990 (US)</td>
<td>Adagen</td>
<td>Pegademase: bovine (PEGylated bovine adenosine deaminase)</td>
<td>SCID with adenosine deaminase deficiency</td>
</tr>
<tr>
<td>1994 (US)</td>
<td>Oncaspar/Elspar</td>
<td>Pegaspargase</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>2000 (EU)</td>
<td>Peg-Intron</td>
<td>rIFN alpha-2b</td>
<td>Chronic hepatitis C</td>
</tr>
<tr>
<td>2000 (EU)</td>
<td>ViraferonPeg</td>
<td>PEGylated rIFN alpha-2b</td>
<td>Chronic hepatitis C</td>
</tr>
<tr>
<td>2002 (EU, US)</td>
<td>Pegasys</td>
<td>IFN alpha-2a</td>
<td>Hepatitis C</td>
</tr>
<tr>
<td>2002 (EU)</td>
<td>Somavert</td>
<td>hGH analog</td>
<td>Acromegaly</td>
</tr>
<tr>
<td>2004 (US)</td>
<td>Macugen</td>
<td>Pegaptanib (VEGF antagonist)</td>
<td>Neovascular (wet) age-related macular degeneration</td>
</tr>
<tr>
<td>2010 (US)</td>
<td>Krystexxa</td>
<td>Pegloticase</td>
<td>Gout</td>
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</table>
The first two molecules to be PEGylated and approved by the FDA for clinical use were pegademase bovine (Adagen®, approved in 1990) and pegasparagase (Oncaspar®, approved in 1994). In the case of Oncaspar®, PEGylation provided distinct benefits: first, an improved pharmacokinetic (PK) profile (increased $T_{1/2}$) resulted in a reduction in the frequency of dosing and increased patient compliance; second, PEGylation had a notable effect on immunogenicity, reducing the rate of ADA formation to 12% from 28% observed with the unmodified protein [61]. The early methods used for PEGylation of proteins were nonselective, resulting in heterogeneously modified drug products with different degrees of PEGylation and inter-batch variability. From these initial products, however, improvements in technology have been achieved that enable more precise, site-specific PEGylation and these more homogeneous products have improved clinical profiles and are more satisfactory to regulatory authorities because they are more consistent and better characterized [60].

An example of a “next-generation” PEGylated protein is Pegasys® (PEGylated interferon alfa-2a). In this product, recombinant alfa-2a interferon is covalently conjugated at a single site to a single-branched PEG chain. Compared to the non-PEGylated protein (Roferon A®), Pegasys® produced a 12- to 135-fold increase in viral clearance in animal models, indicating a substantial improvement in efficacy. In addition, the $T_{1/2}$ of Pegasys® is 80h compared to 5h for Roferon A®, which results in a decrease in clinical dosing frequency from three times per week to once weekly and an attendant improvement in patient compliance [62]. These benefits provide a clear justification for efforts to engineer the native protein and obvious advantages to patients.

**Depot systems**

Another novel protein engineering approach that has been employed to improve the PK of protein therapeutics is the parenteral depot system, which to date has included microspheres, nanospheres, liposomes, and polymeric solutions or gels (examples of approved depot products are shown in Table 1.4). With this approach, the drug is dispersed in a carrier matrix and the matrix is then injected subcutaneously or intramuscularly. Most of the polymers used to deliver proteins in injectable depot products are biocompatible and biodegradable; the depot degrades into biologically acceptable molecules that are metabolized via normal metabolic pathways. In

<table>
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<tr>
<th>Initial approval (year/country)</th>
<th>Brand name</th>
<th>Active compound</th>
<th>Initial indication</th>
</tr>
</thead>
<tbody>
<tr>
<td>1989 (US)</td>
<td>Lupron Depot</td>
<td>Leuprolide acetate</td>
<td>Palliative treatment of advanced prostate cancer</td>
</tr>
<tr>
<td>1989 (US)</td>
<td>Zoladex</td>
<td>Goserelin acetate</td>
<td>Prostate cancer</td>
</tr>
<tr>
<td>1998 (US)</td>
<td>Sandostatin LAR Depot</td>
<td>Octreotide acetate</td>
<td>Acromegaly</td>
</tr>
<tr>
<td>1999 (US)</td>
<td>Nutropin Depot</td>
<td>Somatropin (rDNA origin)</td>
<td>Growth failure due to lack of endogenous growth hormone secretion</td>
</tr>
</tbody>
</table>
fact, polylactide (PLA), polyglycolide (PGA), and poly(lactide-co-glycolide) (PLGA) are considered GRAS (Generally Recognized As Safe) excipients by the US FDA. As the depot breaks down in vivo by chemical and physical processes, the drug is gradually released into the surrounding tissue and/or taken up into the circulation for widespread exposure. Release typically occurs at a nearly constant rate \[63\], which in many cases is critical for efficacy and helps to improve patient compliance because the frequency of injections can be reduced.

Although the depot approach has achieved increased use due to the advantages it offers, it is not without potential cause for concern. One recent publication summarizes a case report of a Japanese patient being treated for prostate cancer with leuprorelin acetate depot who, following a number of injections of the depot over a period of many years, developed life-threatening anaphylaxis; this publication further describes literature reports of an additional five occurrences of anaphylaxis in response to leuprorelin acetate depot administration and three occurrences of anaphylaxis following administration of the related goserelin acetate depot \[64\]. The authors concluded that there was no particular parameter that was able to predict the anaphylactic reactions to these gonadotropin-releasing hormone analog (GnRHa) depots, and they suggest that the drug carrier PLGA may be responsible for the observed reactions. Another interesting safety-related report, which also happens to refer to leuprorelin acetate depot, describes the occurrence of granulomatous reactions in three patients \[65\]. The authors suggest, and a survey of the literature available for other depot-formulated drugs confirms, that these reactions may be specific to leuprorelin acetate itself; however, these safety-related observations are interesting nonetheless and illustrate the point that continued vigilance is warranted as biotechnology drug delivery technology continues to evolve.

**Pulmonary delivery**

Therapeutic peptides and proteins currently must be administered parenterally (i.e. intravenously, intramuscularly, subcutaneously). Were they to be administered orally, a vast system of catabolic enzymes present in the gastrointestinal tract would rapidly degrade these drugs into their component amino acids, rendering them ineffective. In addition, because of their large size, therapeutic proteins have a limited ability to diffuse through the cellular membranes in the gut. However, parenteral administration of protein therapeutics is invasive and places demands on the patient for convenience and compliance. Challenges encountered during the development and use of therapeutic proteins lead to more advanced protein engineering to overcome limitations and improve clinical utility.

Many small-molecule drugs are administered via inhalation, and this route is a potential alternative to conventional parenteral administration for the delivery of biotherapeutics. Pulmonary drug delivery is attractive because the lungs have a large surface area, a thin alveolar membrane which enables rapid absorption, and an absence of first-pass metabolism. In recent years, studies on pulmonary application of metabolically active hormones (insulin, calcitonin, growth hormone, somatostatin, TSH, and FSH), growth factors (G-CSF and GM-CSF), interleukins, and heparin have been performed. Pulmonary delivery of insulin began to be investigated as early as the 1920s \[66\]; however, it was decades before the technology necessary to facilitate delivery via this route became available. In the early 1990s, the first clinical trials of inhaled insulin were conducted. These efforts culminated in the approval of Exubera® by the US FDA in 2006. However, only a short time after approval
was granted, the manufacturer (Pfizer) announced that it would stop selling the product for financial reasons related to poor patient and physician uptake of the product and technology. Factors that undoubtedly diminished the success of this therapy were the unwieldiness of the device, the lack of clinical superiority over available rapid insulin analogs [67], and the requirement that patients who were current smokers or who had asthma, chronic obstructive pulmonary disease, or other lung disease should not receive Exubera® [68]. In addition, a potential link between Exubera® administration and the development of lung cancer was recognized based on data derived from clinical trials, where six cases of lung cancer were noted in 4740 Exubera®-treated patients compared to one case in the 4292 control patients [69]. These findings also caused discontinuation of the development of inhaled insulin products by other pharmaceutical companies (Novo Nordisk, Lilly). However, the investigation of an inhaled form of insulin with the development of Technosphere® insulin (Afrezza®) has been undertaken (Mannkind Corporation). To date, Afrezza® has been well tolerated by healthy volunteers as well as by diabetes patients participating in clinical trials [70]. Nevertheless, many concerns related to Exubera®, namely the effect of an inhaled protein product on measures of lung function as well as long-term safety, will certainly remain critical to the development of any novel biologic therapies utilizing this route of administration.

Monoclonal Antibodies and their Modifications

There has been a major evolution in mAb design and structure since their introduction to clinical practice. First-generation products were hybridoma-derived murine antibodies, which are produced from the fusion of immortalized myeloma cells with B cells from immunized mice. These early mAbs had limited therapeutic potential given their propensity for immunogenicity and immune complex formation. Advances in chimerization and humanization technologies have dramatically improved the performance of mAbs and more recently the use of human transgenic and phage display technologies have allowed for the development of fully human mAbs. Although immunogenicity has been greatly reduced by these progressive modifications, the potential to induce immune responses in patients remains a significant concern and efforts continue in identifying product and formulation attributes that can decrease immunogenicity. To date, all licensed mAb therapies have been of the IgG class and most have been of the IgG1 subclass. A list of currently approved mAb therapeutics is shown in Table 1.5. For oncology indications and indications where cells are targeted for deletion, the IgG1 subclass has been favored given its superiority in initiating ADCC and CDC. For targets where these effector functions are not desired (e.g. soluble targets), IgG4 and more recently IgG2 subclasses have often been pursued although there are now modifications to IgG1 that effectively knock out effector functions from this framework of mAbs. As new technologies emerge, opportunities to improve attributes and performance of mAbs are being explored to enhance their therapeutic potential. Many novel therapeutic candidates employ modifications to improve PK or to improve efficacy and/or safety by a variety of means including optimization of effector functions, i.e. inclusion of drug payloads (e.g. antibody–drug conjugates), and receptor-mediated transport across the blood–brain barrier, in addition to unique strategies to engage the immune system in cellular targeting therapies.
### TABLE 1.5 Therapeutic Monoclonal Antibodies Approved in the US and EU (Approved through March 2012)

<table>
<thead>
<tr>
<th>Year approved by FDA (EU)</th>
<th>Generic name</th>
<th>Brand name</th>
<th>Antibody type</th>
<th>Target</th>
<th>Initial indication(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1986 (1986)</td>
<td>Muromonab-CD3</td>
<td>Orthoclone OKT3</td>
<td>Mouse IgG2a</td>
<td>CD3</td>
<td>Organ transplant</td>
</tr>
<tr>
<td>1998 (1999)</td>
<td>Infliximab</td>
<td>Remicade</td>
<td>Chimeric IgG1</td>
<td>TNFα</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>1999 (2000)</td>
<td>Trastuzumab</td>
<td>Herceptin</td>
<td>Humanized IgG1</td>
<td>HER2</td>
<td>Breast cancer</td>
</tr>
<tr>
<td>2000 (NA)</td>
<td>Gemtuzumab</td>
<td>Mylotarg</td>
<td>Humanized IgG4</td>
<td>CD33</td>
<td>Acute myelogenous leukemia</td>
</tr>
<tr>
<td>2001 (2001)</td>
<td>Alemtuzumab</td>
<td>Campath</td>
<td>Humanized IgG1</td>
<td>CD52</td>
<td>Chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>2002 (2003)</td>
<td>Adalimumab</td>
<td>Humira</td>
<td>Human IgG1</td>
<td>TNFα</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>2003 (NA)</td>
<td>Tositumomab</td>
<td>Bexxar</td>
<td>Mouse IgG2a</td>
<td>CD20</td>
<td>Follicular lymphoma</td>
</tr>
<tr>
<td>2003 (2005)</td>
<td>Omalizumab</td>
<td>Xolair</td>
<td>Humanized IgG1</td>
<td>IgE</td>
<td>Asthma</td>
</tr>
<tr>
<td>2004 (2005)</td>
<td>Bevacizumab</td>
<td>Avastin</td>
<td>Humanized IgG1</td>
<td>VEGF-A</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>2004 (2006)</td>
<td>Natalizumab</td>
<td>Tysabri</td>
<td>Humanized IgG4</td>
<td>A4-integrin</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>2006 (2007)</td>
<td>Ranibizumab</td>
<td>Lucentis</td>
<td>Humanized IgG1 Fab</td>
<td>VEGF-A</td>
<td>Macular degeneration</td>
</tr>
<tr>
<td>2008 (2009)</td>
<td>Certolizumab</td>
<td>Cimzia</td>
<td>Humanized Fab</td>
<td>TNFα</td>
<td>Crohn’s disease</td>
</tr>
<tr>
<td>2009 (2009)</td>
<td>Canakinumab</td>
<td>Ilaris</td>
<td>Human IgG1</td>
<td>IL-1</td>
<td>Muckle-Wells syndrome</td>
</tr>
</tbody>
</table>
**Enhanced effector functions**

Effector functions (i.e., ADCC and/or CDC) often play a prominent role in the pharmacologic activity of mAbs, and this has been particularly true for many of the tumor-targeting therapies. Rituximab (anti-CD20 mAb), which is currently used in the treatment of non-Hodgkin’s lymphoma and other malignancies, has been a highly successful therapeutic agent that has revolutionized cancer treatment. However, the potential exists to improve clinical benefit and there are many strategies currently being pursued to improve efficacy of this and other B-cell targeting therapies [71]. In the case of rituximab, ADCC, CDC, and receptor-mediated apoptosis are known to contribute to therapeutic cytotoxicity and modifications to improve effector function and/or receptor targeting are promising strategies that are currently under evaluation. FcγRIIIa is found on natural killer (NK) cells, monocytes/macrophages, and dendritic cells, and engagement of the Fc fragment of the mAb with FcγRIIIa is known to be critical for eliciting ADCC activity, particularly through NK cells. Many novel approaches to mAb design aim to increase ADCC activity by increasing affinity for this receptor. Glycosylation of Asn297 is needed for Fc-mediated signaling through FcγRIIIa and removal of fucose from the Fc-associated biantennary glycan core has been shown to result in a 10- to 50-fold increase in binding affinity to FcγRIIIa relative to native IgG1. This strategy has been exploited with recent clinical candidates to boost both in vitro ADCC and in vivo efficacy [71–74]. Two specific ways to accomplish this goal involve expression of β1-4-N-acetylglucosaminyltransferase III
(GnTIII) in CHO cells to promote addition of bisecting N-acetylglucosamine residues and by knocking out α(1–6) fucosyl transferase activity, both of which result in afucosylation of Asn297-linked oligosaccharides [75,76]. However, beyond increasing therapeutic potency, this approach also has the potential to impact nontarget or bystander tissues given the exaggerated impact on effector functions, thus necessitating careful preclinical evaluation prior to entry into clinical trials. Alternatively, in therapeutic approaches where ADCC and CDC are not desirable attributes, effector functions can be knocked out with a variety of approaches including full aglycosylation of IgG1 or IgG3, or use of IgG2 or IgG4 constructs, which naturally possess limited effector functions [77].

More recently, engineering efforts have included strategies in which other relevant immune cell types such as macrophages are targeted, which may involve modifications to augment Fcγ receptor binding. Notably, improved binding to FcγRIIa (an activating pathway) relative to FcγRIIb (inhibitory) can improve macrophage-mediated phagocytosis and for some tumor indications where myeloid lineage cells are thought to play an important role this could be an advantageous approach [78,79]. In addition, chimeric human IgGs have been developed that swap out key regions of IgG3 to enhance complement recruitment while maintaining optimal ADCC activity [80,81] and specific mutations to increase IgG binding to C1q, the trigger for classical complement activation, allow for the enhancement of Fc-mediated complement activity and CDC [82].

MAbs can act as antagonists (i.e. blocking agents) or inverse agonists (i.e. agents that induce a response opposite to that of an agonist) and typically bind cellular receptors in a fashion that minimizes intracellular signaling or prevents cross-linking mediated signaling. The typical mAbs have two Fab arms that bind to the same therapeutic target, either cellular receptor or soluble ligand, and thus are referred to as bivalent mAbs. Recently, a different type of monovalent mAb has been developed. Onartuzumab (METMab) is an example of a unique one-armed antibody that was specifically designed as a monovalent antibody to avoid agonistic activity that may occur when a bivalent antibody binds two MET molecules. Onartuzumab binds to MET with high specificity to prevent binding of its ligand Hepatocyte Growth Factor (HGF), and subsequent downstream signaling through the MET receptor, a key mediator or tumor growth. The monovalent binding was shown to be superior to binding by bivalent antibodies for inhibition of MET signaling and this monovalent therapeutic mAb candidate is currently in Phase III trials for non-small cell lung cancer [83].

**Bispecific antibodies**

In general, human (bivalent) IgGs are monospecific because both Fab arms have identical heavy and light chain structure. The one notable exception is IgG4, which contains considerable heterogeneity in Fab composition due to dynamic Fab arm exchange. IgG4 therapeutics, such as natalizumab, which targets the α4 arm of α4β1 and α4β7 integrins, have been shown to undergo dynamic Fab arm exchange with endogenous polyclonal IgG4 in clinical trial patients [84]. More recent clinical IgG4 candidates have incorporated a serine to proline substitution (S228P) to stabilize the hinge region, which is more analogous to the sequence found in IgG1 [85,86]. In contrast to conventional monospecific mAbs, therapeutic bispecific mAbs are designed to target two distinct epitopes, which can allow for binding to multiple targets or enable specific cell–cell interactions that would not be possible with monospecific mAbs. Currently, there are many platforms under development that utilize cross-linked antibody fragments or fully functional bispecific mAbs that are engineered with intact effector functions. Fc-mediated dimerization
has also been used to produce bispecific tetravalent antibodies that contain two binding sites for each of two targets [87]. One novel technology generates what is referred to as a dual action antibody, where each of two identical Fabs maintains the ability to bind two distinct epitopes. Following normal affinity maturation for a specific target, a repertoire of antibody variants with mutations in the light chain complementarity determining region (CDR) are screened against a secondary target to optimize and maintain affinity to both distinct targets [88].

Even more unique engineering of bispecific mAbs is exemplified by a strategy in which both tumor cell surface antigens and T cells involved in the antitumor response are linked by a bispecific T-cell engager (BiTE). BiTE antibodies are constructed from two short, flexibly linked, single-chain antibodies, which engage both CD3-bearing T lymphocytes and numerous targets expressed on tumor cells, such as CD19, EpCAM, and CEA [89]. Blinatumomab binds both CD19 antigen and the T-cell receptor (CD3) and when engaged to both targets, transiently activates T cells, thereby directly targeting cytolytic potential against CD19-bearing cells. One remarkable aspect of this approach is that blinatumomab has demonstrated partial and complete tumor remission at doses as low as 0.015 mg, which is many orders of magnitude lower than the dose required for conventional mAb tumor-targeting therapies [90]. However, that efficacy comes at a price as cytokine release-associated toxicities must be carefully managed. Catumaxomab, which is currently licensed in the EU for malignant ascites, contains Fabs that bind both CD3 antigen and EpCAM; however unlike the BiTE technology, this MAb also retains an Fc region with intact effector functions and thus is often referred to as a trifunctional antibody. This approach allows for tumor destruction by T cell-mediated lysis, ADCC, cell-mediated cytotoxicity and phagocytosis [91]. Similar approaches include ertumaxomab, which selectively cross-links CD3-expressing T cells and HER-2-neu-expressing tumor cells.

Antibody–Drug Conjugates

The concept of delivering cytotoxic payloads to specific targets using the high affinity, specificity, and long half-life of antibodies has been around for several decades. Early attempts were hampered by the instability of linkers, immunogenic potential, and inappropriate specificity of target/bystander cytotoxicity [92,93]. The first antibody–drug conjugate (ADC) to win market approval in the US was gemtuzumab ozogamicin, which is derived from an anti-CD33 IgG4 that has been covalently attached to a cytotoxic payload N-acetyl-γ calicheamicin. This was approved by the FDA in 2000 for the indication of acute myelocytic leukemia (AML), although it was subsequently withdrawn from the market. Since this early success there has been an explosion of ADC candidates that have entered clinical trials and the complexity of linkers, drug payloads and mAb targets continues to expand (reviewed fully in Chapter 5).

Given the limitations of current linker systems, bystander toxicities, and the metabolic fate of cytotoxic drug components, the administration of ADCs generally results in drug-limiting toxicities. Therefore, preclinical safety programs that support clinical candidates often need to identify target organ toxicities, appropriate dosing regimens, and establish appropriate therapeutic indices to allow for successful entry into clinical trials, and current strategies are discussed in detail in Chapter 5.

Improved pharmacokinetics

One of the early strategies to extend the half-life of mAbs or mAb fragments was to enlarge the hydrodynamic diameter by addition of bulky groups such as by chemical conjugation with
polyethylene glycol chains (PEGylation). Certolizumab, a PEGylated Fab' fragment, was the first to be approved by the FDA for clinical use in 2008. Recent strategies have also pursued structural changes to optimize affinity to the neonatal Fc receptor (FcRn). This receptor plays a critical role in capturing pinocytosed IgG for recycling back to the vascular compartment, thus sparing antibodies from endosomal catabolysis. It has been hypothesized that if an optimal binding affinity at the low pH in endosomes can be achieved while minimizing affinity at neutral pH then improvements in mAb recycling can be attained, but many have argued that this relationship may prove more complex [94,95]. Nonetheless, improvements in pharmacokinetics in non-human primates have been achieved with several specific Fc mutations that were designed to modify FcRn binding, including N434A, T250Q/M428L, and S254T/T256E [43,96,97].

**Immunoliposomes**

Engineered liposomes have proven useful for targeted drug delivery in oncology settings as liposomes preferentially accumulate in tumors and inflammatory tissue by passive diffusion due to leaky vasculature and lack of effective lymphatic drainage [98,99]. Antibodies or antibody fragments that are attached to liposomes to form immunoliposome conjugates offer further potential to enhance accumulation of drug payloads in tumor tissues. MCC-465 was the first therapeutic immunoliposome to enter clinical trials and a Phase I study in support of colorectal, stomach and other solid tumor indications has been completed [100,101]. This candidate comprises doxorubicin that is encapsulated in a PEG immunoliposome tagged with an antigen-specific F(ab')2 fragment. Another candidate that has completed a Phase I trial for solid tumors is SGT-53. This immunoliposome encapsulates a plasmid coding for the tumor suppressor p53 gene and incorporates a single-chain antibody fragment (anti-TfR scFv) to target the transferrin receptor. Other candidates that have shown promise in preclinical models include immunoliposomes that specifically target IGF1 receptor, anti-cMet, anti-ErbB2, and HER2 [102–105].

**Crossing the blood–brain barrier**

The blood–brain barrier (BBB) serves an essential role in controlling entry of required substances and preventing transit of potentially deleterious molecules into the brain. It is comprised of brain capillary endothelial cells that are distinct in structure and function from other endothelial cells of the body. Molecules able to cross the BBB do so via lipid-mediated diffusion or receptor-mediated transport (RMT) [106]. The delivery of both small- and large-molecule therapeutics to the brain to treat diseases of the central nervous system is limited by the presence and structure of the BBB. For small-molecule therapeutics to effectively enter the brain, they must have a low molecular weight (<400 Da) and high lipid solubility/low hydrogen bonding capacity [106]. Although the neonatal Fc receptor (FcRn) has been shown to be present at the BBB [107] it appears to regulate transcytosis of IgG from the brain to the blood, and not the other way around [108]. However, mAbs have been shown to be able to access the brain by specifically targeting receptors present on the luminal membrane of the brain capillary endothelial cell [109]. A number of endogenous proteins enter the brain via specific transporters (i.e. insulin, leptin, transferrin), and this RMT system has been used for the delivery of biopharmaceuticals to the brain [110]. One approach, known as the molecular Trojan horse, utilizes fusion protein technology to couple a therapeutic to a peptidomimetic mAb targeting one of the brain-specific transporters. Therapeutic efficacy using this approach...
has been demonstrated in a mouse model of Parkinson’s disease with either tumor necrosis factor receptor [111] or erythropoietin [112] as the therapeutic coupled to a mAb against the transferrin receptor (TfR) (the so-called Trojan horse). An extension of the Trojan horse technology utilizes a targeting ligand directed against an endogenous BBB receptor coupled to a PEGylated liposome encapsulating plasmid DNA or RNA to direct a gene therapy to the brain [113]. A notable advance in understanding the mechanism of transport of therapeutic antibodies using RMT recognized the importance of binding affinity of the antibody for the transport protein. Antibodies with reduced affinity for the TfR crossed the BBB more effectively than those with higher affinity because the high-affinity antibodies, rather than being released into brain parenchyma, remained associated with the BBB [114]. The potential therapeutic value of this approach was confirmed by the generation of a bispecific anti-TfR/BACE (β-secretase) antibody; the low-affinity anti-TfR arm of the antibody facilitated transit into the brain, where the inhibitory activity of the BACE arm effectively reduced the levels of brain amyloid-β [114].

Fusion Protein Constructs

Following on the success of therapeutic mAbs, another class of biopharmaceutical drugs, the fusion proteins, has been developed and examples of approved fusion protein products are shown in Table 1.6. Although protein therapeutics typically have a long

<table>
<thead>
<tr>
<th>Initial approval (year/country)</th>
<th>Brand name</th>
<th>Active compound</th>
<th>Initial indication</th>
<th>Distributor</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998 (US) Enbrel</td>
<td>Etanercept (75kDa soluble extracellular domain of tumor necrosis factor (TNF) receptor II fused to human IgG1 Fc)</td>
<td>Rheumatoid arthritis</td>
<td>Amgen/Pfizer</td>
<td></td>
</tr>
<tr>
<td>1999 (US) Ontak</td>
<td>Fusion of IL-2 with diphtheria toxin</td>
<td>Cutaneous T-cell lymphoma</td>
<td>Esai</td>
<td></td>
</tr>
<tr>
<td>2003 (US) Amevive</td>
<td>Alefacept (extracellular human leukocyte functional antigen 3-IgG fusion protein)</td>
<td>Plaque psoriasis</td>
<td>Biogen Idec</td>
<td></td>
</tr>
<tr>
<td>2005 (US) Orencia</td>
<td>Abatacept (CTLA4-Fc)</td>
<td>Rheumatoid arthritis</td>
<td>Bristol Myers Squibb</td>
<td></td>
</tr>
<tr>
<td>2008 (US) Nplate</td>
<td>Romiplostim (dimeric Fc fusion protein with each monomer consisting of two thrombopoietin receptor-binding domains)</td>
<td>Thrombocytopenia</td>
<td>Amgen</td>
<td></td>
</tr>
<tr>
<td>2011 (US) Nulojix</td>
<td>Belatacept (extracellular CTLA-4–Fc fusion protein)</td>
<td>Prophylaxis of organ rejection (kidney transplant)</td>
<td>Bristol Myers Squibb</td>
<td></td>
</tr>
<tr>
<td>2011 (US) Eylea</td>
<td>Aflibercept (VEGFR1/VEGFR2–Fc fusion protein)</td>
<td>Neovascular (wet age-related macular degeneration (AMD)</td>
<td>Sanofi-Aventis/Regeneron Pharmaceuticals</td>
<td></td>
</tr>
</tbody>
</table>
plasma residence time compared with small molecules, the requirement for parenteral administration impacts patient inconvenience for chronic therapy. Another strategy for improving drug half-life is the fusion of the therapeutic protein to a larger protein such as the Fc domain of an immunoglobulin, albumin, transferrin, or an immunotoxin such as diphtheria toxin [115].

In the case of the Fc fusion proteins, half-lives are extended via interaction of the fusion protein with the neonatal Fc receptor (FcRn) which exhibits pH-dependent binding. Fc-proteins are internalized via pinocytosis and interact with the FcRn within intracellular endosomes that have low pH. FcRn-bound fusion proteins are recycled back to the cell membrane and at the neutral pH of the cell membrane the fusion protein is released back into circulation [116], thereby prolonging serum half-life. An additional benefit to the fusion of a protein with an Ig Fc region is that the functional activity of the Fc region (i.e. ADCC and CDC) increases effectiveness of such constructs. In the case of albumin fusion proteins, the primary advantage gained is an increase in serum half-life as albumin is the most abundant plasma protein and has an average half-life of 19 days [117]. Similarly, the transferrin fusion proteins seek to take advantage of the long half-life (14–17 days) of transferrin (Tf). Proof of therapeutic principle achieved for the Tf-fusion proteins (e.g. GLP-1-Tf (glucacon-like peptide-1 fused to transferrin) and EX-4-Tf (exendin-4 fused to transferrin)) confirm the ability to obtain the pharmacological effects of the native proteins with extended durations of action [118].

Immunotoxin fusion proteins typically utilize either plant toxins (e.g. saporin, bouganin, gelonin, pokeweav antiviral protein, or bryodin 1) or class II ribosome-inactivating proteins (ricin, abrin, modeccin, or mistletoe lectin). Bacteria present another source of toxins, with the most frequently utilized being Pseudomonas endotoxin and diphtheria toxin [115]. The plant and bacterial toxins function by inhibiting elongation factor 2 (EF2) from interacting with the 60s ribosome and thereby inhibit protein synthesis which eventually leads to cell death. Although immunotoxins are favored because of their targeted toxicity and limited side effects, they are highly immunogenic because of their “non-human” origin. This could be a significant limiting factor in their overall utility as biotherapeutics. An additional potential limitation can be found in countries where most of the population has been immunized against diphtheria; vaccinated individuals will have circulating antibodies against diphtheria toxin which will result in the neutralization of diphtheria toxin-based immunotoxins. A novel immunotoxin approach designed to overcome many of these limitations combines an endogenous protein of human origin with cytotoxic activity, such as a proapoptotic protein (e.g. Bax, sTRAIL, granzyme B, FasL), with an mAb specific for a tumor-expressed antigen (e.g. VEGF, EGFR, ErbB2) [119]. These conjugates should target tumor cells preferentially by virtue of the overexpression of the tumor-expressed antigen, and should have limited peripheral toxicity as a result.

Other classes of fusion proteins include the cytokine fusion proteins, which fuse IL-2, IL-12, or TNF with an antibody against tumor cell surface antigens for use in oncology indications. These drugs are theoretically more effective than administering the individual proteins in combination; however, to date all such compounds are still in clinical development [115]. Additional novel approaches will undoubtedly be used as protein engineering efforts to fuse independent proteins are developed in an ever-broader range of clinical indications.
COMPARISON OF SMALL-MOLECULE DRUGS TO BIOPHARMACEUTICALS

While about 200 biopharmaceutical drugs have been approved and used in human medicine, approximately 1100–1200 chemical or small-molecule drugs have been approved by the FDA since 1950 [120,121], with the most recent number at time of press being as high as 1447 according to the DrugBank database [122]. The long history of small molecules (i.e. low molecular weight organic compounds) has paralleled advances in chemistry, pharmacology, and biochemistry that enabled interdisciplinary approaches as described by Drews in 2000 [123]. One of the first small molecules isolated for medicinal use was morphine, extracted from the opium plant in 1815 by F. W. Sertürner; this provided technical capability for the isolation of additional small-molecule pharmaceuticals [123]. The intensified isolation and purification of a variety of pharmacologically active compounds from medicinal plants over time was enabled by advances in analytical chemistry, while development of new chemical methods facilitated the generation of analogs through controlled chemical reactions.

The advent of new technologies and advances in sciences such as molecular biology have increased the rate at which chemical structures are generated and screened; high-throughput in vitro assays against potential drug targets has reached approximately 50 million compounds annually. The ability to direct the specific binding of a small-molecule drug to a target protein or enzyme, with optimization of physical, chemical, and delivery attributes, has further increased the numbers of compounds in drug discovery. However, an analysis of the number of potential small-molecule drug candidates coupled with disease-related genes suggests that although high-throughput screening is at an all-time high, the number of successful new chemical entities as drugs represent only an average of four novel therapeutic targets annually [124]. In this section, we compare the properties of small-molecule drugs to those of biopharmaceuticals and discuss their similarities and differences in drug development.

Properties of Chemical and Biologic Drug Candidates

In pharmaceutical development, a small molecule is defined as a chemically synthesized molecular entity with a low molecular weight of approximately 500 Da ideally, but generally less than 1000 Da [125]. In contrast, the protein components of biopharmaceuticals have a molecular weight ranging between 1000 and 30,000 Da for peptides and proteins, and over 100,000 Da for mAbs; these are therefore termed “large molecules.”

The synthesis of small-molecule drugs is tightly controlled, and both active pharmaceutical ingredient (API) and the final drug product are regulated by health authorities. The final drug product contains a high-purity API with set limits dependent upon the stage of development for an allowable percentage of impurities, genotoxic impurities, and residual solvents [126–128]. Similarly, production of large molecules manufactured from various host cell systems are tightly controlled during the manufacturing process and represent more complex, multi-ordered (primary, secondary, tertiary) structures compared with small molecules [129].

The drug-like properties of a chemical compound are defined by its structure, such as molecular weight, hydrogen bonding, lipophilicity, pKₐ, polar surface area, shape and reactivity; by its physicochemical characteristics, such as solubility, permeability and chemical stability; and
by its biochemical attributes such as metabolism, drug–drug interactions, and cellular uptake and transport [130,131]. These structural, physicochemical, and biochemical properties form the basis of a compound’s ability to enter a cell and/or nuclear membrane by passive diffusion or active transport and bind a specific biological target to elicit its intended pharmacologic action. Most commercial small molecules being developed today are optimized for oral administration. Based on Lipinski’s “rule of five” [132] for orally bioavailable small-molecule drugs, poor solubility or permeability occurs when molecular mass >500 Da, lipophilicity is high (calculated logP >5), there are more than five hydrogen-bond donors, and the sum of nitrogen and oxygen atoms in a molecule is >10. However, subsequent analysis has shown that only 51% of FDA-approved small-molecule drugs are orally administered and have followed Lipinski’s “rule of five,” [121] suggesting that other routes of administration including parental, inhalation, dermal/topical, or parallel efforts using a multifaceted approach can be explored by the drug development scientist [133]. However, the properties that make a small molecule desirable for its pharmacological potential may also contribute to its lack of specificity or selectivity for interactions with the intended therapeutic target. Small molecules can distribute broadly to other tissues, including normal cells, and cause off-target toxicity. In contrast, a biopharmaceutical is highly specific in binding to the intended pharmacological target, nonspecific cellular uptake is possible via Fc receptors (for antibodies), or pinocytosis. Biopharmaceuticals do not produce active metabolites as they are catabolized to natural amino acids and cleared via the kidneys (like endogenous proteins). They do not undergo detoxification in the liver or other tissues, unlike chemicals (drugs and others) to which the human body is exposed. Because biopharmaceuticals do not bind to plasma proteins, classical cytochrome P450-mediated drug–drug interactions are not of concern. In contrast, the inhibition or induction of cytochrome P450 enzymes, most notably P450 3A4, the human enzyme involved in metabolism of over 50% of drugs, is a prominent consideration in the development of new chemical entities. Great effort is taken to design this liability out of drug candidates during the lead optimization phase in drug discovery. In addition, off-target toxicities may be induced by potential interaction of a small molecule with drug transporters (e.g. P-glycoprotein transporters) in various tissues such as brain, small intestine, and kidney acting as a drug substrate which can result in its greater concentrations in those tissues [131].

Both small-molecule and biopharmaceutical drugs have sought to improve pharmacokinetic properties during discovery and development, thereby conferring the greatest possible therapeutic benefit to patients. In contrast to biopharmaceuticals for which a long half-life is desirable to reduce injection frequency, low to moderate clearance, and thus longer half-life for chemical drugs can be highly undesirable, as it may lead to interaction with other drugs rendering one or both of them less efficacious and/or more toxic [131]. Given the potential for off-target toxicity, a longer half-life may prolong undesirable side effects. Small molecules can fail due to poor bioavailability (i.e. systemic exposure in blood plasma after oral administration). In fact, a decrease in bioavailability from 39% to 10% between 1988 and 2000 has been reported for chemical compounds [134]. Biopharmaceuticals differ in this aspect as they often have bioavailability of 80–100% for subcutaneous and intravenous administration, respectively. For both small molecules and biologics, prior to first-in-human (FIH) clinical trials the human efficacious dose, exposure, and key pharmacokinetic parameters can be predicted from preclinical pharmacology and toxicology models using modeling and simulation techniques.
Nonclinical Species for Toxicology Testing

Early in the era of biotechnology, it became apparent that characterizing the toxicity of biopharmaceuticals in support of clinical trials, and ultimately marketing approvals, would require a fresh approach. Since biotechnology-derived therapeutics are designed to be highly human specific, as defined by protein sequence homology, target binding affinity, and functional potency, translating these activities into pharmacology and toxicology animal models requires screening for these attributes *in vitro* or *ex vivo* across multiple animal species [135]. The high degree of species specificity observed with human protein therapeutics combined with species differences in biology resulted in the selection of animal models for toxicology that were pharmacologically responsive. This was a departure from criteria used to select animal models for traditional small-molecule pharmaceuticals that relied mainly on pharmacokinetic properties (primarily bioavailability) and metabolic profiling [136].

The selection of a relevant toxicology species for a biopharmaceutical is based on demonstration of the expression of the target antigen with similar activity to humans. A variety of techniques (e.g. immunochemical and functional tests) need to be developed to identify a relevant species using molecule (biopharmaceutical candidate)-specific reagents and assays. As evidenced by the interferons [137], failure to assess toxicity in a pharmacologically relevant animal model can create a false sense of security, when no toxicity is elicited due to species specificity and/or the generation of neutralizing antibodies in the test species. Pharmacologic activity is a key consideration in the selection of appropriate nonclinical species for the evaluation of biotherapeutics. For a species to be relevant for nonclinical toxicology evaluation, the target antigen should be expressed and distribution should be similar to humans, and the biotherapeutic must be able to bind to the target and elicit effects that parallel those elicited in humans. If two relevant species (one rodent and one non-rodent) are identified as pharmacologically relevant, both species should be used in toxicology studies. Because of the high degree of species specificity, many biopharmaceuticals cross-react in human and non-human primates (NHP) only; thus NHP would be identified as the relevant toxicology species.

In some cases, there are no relevant animal models or the only species with target binding/activity comparable to that of human is the chimpanzee. In those instances, the criteria for use of the chimpanzee in biomedical research is highly restricted and of limited value. Chimpanzee use may be justified only after meeting the following criteria: (1) no other suitable models exist (e.g. *in vitro* or non-human *in vivo*); (2) the research in question could not ethically be performed on human subjects (i.e. with microdosing); and (3) important advancements to prevent, control, and/or treat life-threatening or debilitating conditions would be significantly slowed [138]. For humanized mAbs these criteria are generally not met, as advances in protein/antibody engineering have made it possible to generate proteins with desired attributes such as cross-species reactivity. In some cases, a homologous or surrogate (often rodent) molecule may be considered as the only option to identify potential toxicity related to modulating the target in question. This approach requires careful examination of the analogous product’s properties in comparison to that of the clinical candidate, as well as thorough characterization of target expression and biology in the surrogate nonclinical species prior to use of the surrogate molecule in safety assessment studies [125].

For all small-molecule drug candidates, two animal species, rodent and non-rodent (historically the rat and dog), are required to be tested in toxicology studies. The understanding
of species relevance is based on a comparison to human metabolite profiling in liver microsomes and/or hepatocytes across a number of test species (i.e. mouse, rat, dog, or monkey). These test systems are well established and can be readily applied for the assessment of small molecules. Toxicology studies in two different animal species increases the probability that any toxicities observed in both species (rather than just one) would be more predictive of potential reactions in a clinical setting and would provide more adequate hazard identification and safety margins prior to the initiation of clinical trials. Unlike for biopharmaceuticals, evidence of pharmacologic or pharmacodynamic (PD) activity in nonclinical species is not always required for chemical entities. However, the demonstration of PD activity has become increasingly desirable in at least one species during the assessment of toxicity for small molecules to better understand dosimetry relationships between drug activity and toxicity. The benefit of establishing relevance of nonclinical species based on PD rather human-specific metabolic profile for toxicology testing of small molecules that is consistent with the approach taken for biopharmaceutical development has been demonstrated for one reported small-molecule kinase inhibitor.

For example, during the toxicity assessment of p38α mitogen-activated protein kinase (MAPK) inhibitors of varying potency, structural diversity, or selectivity against other isoforms (β, δ, and γ) and serine/threonine kinases, the beagle dog was selected for nonclinical safety studies based on metabolic profile. The dog appeared to be quite sensitive to p38 MAPK inhibitors due to higher expression of the target on B cells, and dogs presented with acute gastrointestinal and lymphoid toxicity attributed to B lymphocyte depletion that was not observed in humans or other species (mouse, rat, cynomolgus monkey) [139]. Thus, although the dog may have represented an adequate toxicology species based on comparable human metabolite profile, the dog-specific toxicity findings following administration of p38 MAPK inhibitors had limited predictive power for an assessment of human risk.

Principles of Toxicology Evaluation for Small-molecule Drugs and Biopharmaceuticals

The introduction of biotechnology-derived pharmaceuticals produced new models and strategies for characterizing the safety profile of these products. These approaches were pioneered by industry and health authority scientists and resulted in a “case-by-case” recommendation to the characterization of the nonclinical toxicologic profile of biotechnology products [125,140], culminating in the publication of harmonized regulatory guidance [141,142]. Although the approaches are different, the principles underlying toxicology testing for both small-molecule and biopharmaceutical drugs have the same goal: to rigorously characterize the drug candidate’s safety profile to enable entry into clinical trials.

In determining a starting dose for first-in-human (FIH) studies a no-observable-adverse-effect level (NOAEL) established in toxicology studies is generally considered for both small-molecule and biopharmaceutical drugs. Small-molecule therapeutics for oncology indications determine the severely toxic dose levels in 10% of rodents and/or a highest non-severely toxic dose level in non-rodents as part of the FIH dose selection algorithm [143,144]. For some biopharmaceuticals (e.g. high-risk targets) the determination of a pharmacologically active dose (PAD) or a minimum anticipated biological effect level (MABEL) is recommended [125].
The rationale for selection of high doses to be tested in toxicology studies can be different for small-molecule and biopharmaceutical development programs; using either a maximum tolerated dose (MTD), or the maximum feasible dose (based on both the drug concentration and highest acceptable dose volume for the species and route of administration), to achieve a minimum of 10-fold higher exposure in animals than the highest estimated exposure in humans.

The duration of toxicology studies to support FIH or early clinical trials should mirror that planned for clinical development and are first assessed in an IND-enabling program (e.g. duration matched in nonclinical studies to the protocol-specified duration in the clinic, with an extension for oncology settings based on risk/benefit evaluation). Where small molecules and biopharmaceuticals differ is in the conduct of chronic toxicology studies to support chronic administration in the clinic. Typically for a small molecule, non-oncology indication, chronic studies include six-month rodent studies and nine-month non-rodent studies are recommended [145]. In contrast, six-month chronic studies, regardless of species, are generally considered adequate for all chronically administered biopharmaceuticals [146].

Safety pharmacology assessments intended to address drug effects on vital organs such as the cardiovascular, central nervous, and respiratory systems should be performed for all small-molecule drugs [143,145,147,148]. An in vitro assessment of potential QTc prolongation by determining the concentration at which 50% inhibition of the human potassium (iKr) channel (in vitro hERG assay) occurs is conducted for all small molecules [148]. In contrast, because proteins are too large for the hERG channel this assessment is not relevant for biopharmaceuticals [149]. However, an in vivo assessment of safety pharmacology endpoints, such as functional observational battery tests, and cardiovascular endpoints in telemetry-instrumented animals can be incorporated into general toxicology studies for both small-molecule and biopharmaceutical toxicity studies, resulting in a general reduction of animal usage consistent with the principles of 3Rs (replacement, reduction, refinement).

The standard battery of in vitro and in vivo genetic toxicology assays is warranted for all small molecules based on their ability to pass through cellular and nuclear membranes and interact with DNA. Similarly, genotoxic impurities in a small-molecule drug product may need to be assessed using in silico and/or experimental assays [150,151]. In contrast, biopharmaceuticals do not interact directly with DNA or chromosomal material and are excluded from genotoxicity testing. In addition, for biopharmaceuticals, process-related impurities (residual host cell proteins, fermentation components, leachables, or detergents) are also unlikely to react directly with DNA or other chromosomal material.

Small molecules intended for chronic administration, outside the oncology setting, are assessed for carcinogenic potential in animals in a 2-year rodent bioassay and/or a transgenic mouse model for identification of risks to humans. In general, data from these studies are required at the time of market authorization. A rigorous assessment of the relative merits of the models selected for carcinogenicity testing and doses to be used in such studies should be discussed with health authorities prior to conduct of these studies. For biopharmaceutical drugs, standard rodent carcinogenicity bioassays are generally not appropriate. When there is a cause for concern regarding a biopharmaceutical’s ability to induce proliferative changes, or modulate immune surveillance, other means of assessing potential tumorigenic risk, including a review of pertinent literature, and existing animal and clinical data related to the therapeutic target, should be undertaken. In addition, “incorporation of sensitive indices of cellular proliferation in long-term repeated dose toxicity studies may provide useful information,” according to ICH S6(R1) [142].
The need for a reproductive and developmental toxicity assessment for small-molecule and biopharmaceutical drugs stems from inclusion of women of childbearing potential (WCBP) and men who may father children in clinical studies. It is essential to minimize the risks to the embryo or fetus when including WCBP in clinical trials, and to understand the risks to future fertility. For small molecules, the studies to support inclusion of WCBP are outlined in ICH M3 [145], however there are also options for patients to take appropriate precautions (pregnancy testing, highly effective birth control method, or inclusion in clinical trial after confirmed menstrual period) to prevent pregnancy during clinical trials. Details of the nonclinical study designs are well established and standardized as outlined in ICH S5 [152]. The studies include an evaluation of fertility, where both drug-treated males and drug-treated females are mated to naïve counterparts; embryo–fetal development (EFD) where the drug is administered during organogenesis to assess the risk to the embryo and/or fetus; and pre- and postnatal development (PPND) where the test article is administered to pregnant animals and offspring are assessed for potential developmental impairment. For biopharmaceuticals that are active in rodents and rabbits, both species can be used to assess reproductive and developmental toxicity, however due to different dosing frequency, limited cross-placental transfer and significant concerns for immunogenicity impacting drug exposure in these species, the study design has to be tailored to each biological molecule and often differs from the standardized study designs prescribed for small-molecule drugs [153]. When the biopharmaceutical is only pharmacologically active in the NHP, several approaches can be employed and may consist of separate EFD and PPND studies, or one appropriately designed study that includes dosing from organogenesis to birth, called an enhanced PPND (ePPND) [154]. While the assessment of fertility endpoints for biopharmaceuticals that are pharmacologically active in rodents are feasible, mating studies in NHPs are not practical. If the NHP is the only relevant species, an extended evaluation of the reproductive tract by histopathology and/or relevant hormone levels in sexually mature animals can be incorporated into general chronic toxicity studies as a means of assessing male and female fertility [153].

Toxicology studies uniquely applicable to small molecules include phototoxicity evaluation (in vitro and in vivo) based on the photochemical properties of small molecules’ absorption in the ultraviolet-visible range, distribution to skin and eyes, as well as nonclinical or clinical observations of phototoxicity [143,145,155].

An assessment of immunotoxicity, as described in ICH S8 [156], is focused on small molecules not designed to interact with the immune system, thus the recommended evaluation addresses primarily unintentional immunotoxicity. However, an immunotoxicology assessment is as important for both small and large molecule immunoregulatory agents. In particular, many mAbs for the treatment of inflammatory/autoimmune diseases or cancer, are designed to directly modulate (suppress or stimulate) the immune system. A thorough understanding of the full spectrum of the immunopharmacology of these drug candidates is critical, and has to be considered as part of their toxicology programs. In addition, biopharmaceutical candidates can elicit an immune response (imunogenicity) in animals as they are foreign (human) proteins (antigens) that can induce an antibody response (anti-drug antibodies). Immunogenicity in toxicology studies may have an impact on PK, PD (as discussed in Chapter 4) and/or the toxicity profile and immunogenicity assessment is critical for interpretation of toxicology data. While the incidence or magnitude of the immune response in preclinical species is not predictive of human responses [55], immunogenicity-mediated toxicity should be carefully considered as these effects may result if an immune response is observed in humans.
SUMMARY

Since their introduction into clinical practice in the late 1990s, the first generation of protein therapeutics has demonstrated great utility in a wide array of indications, both acute and chronic, and owe much of their success to their high degree of target specificity. A diverse array of chemical, structural, and formulation modifications to proteins and mAbs has evolved over the last three decades and continues to develop as new molecular modalities to enhance pharmacokinetics, target specificity, signaling and effector functions are designed and tested. It is clear that new advances bring new considerations and challenges. Antibody-drug conjugates, bispecific antibodies, and long-acting protein and antibody constructs offer clinical promise and technical complexity in characterizing their activity and safety. The rapid and substantial accumulation of our understanding of disease biology coupled with advances in protein engineering, genetics, nanotechnology, immunology, and other disciplines ensures continued expansion of the therapeutic potential of biotechnology-derived products.

Although there are differences in the models available to the preclinical scientist to characterize the toxicity of small-molecule pharmaceuticals and biopharmaceuticals, the prevailing scientific objectives of selecting the most appropriate experimental model and designing the most rigorous studies, for the purpose of predicting human risk, are the same. Advances in protein engineering enable the creation of biopharmaceutical constructs designed to improve uniformity, safety, efficacy, or duration of effect. This customization bears some resemblance to optimization of chemical scaffolds during lead optimization with small molecules, where desired drug properties are engineered in and toxicities are engineered out. While species specificity for small-molecule pharmaceuticals has typically been limited to comparison of metabolite profiles, a better understanding of the relevance of the pathway of interest in the test species along with a functional readout has been shown to be valuable in selecting the most appropriate toxicology model, which is consistent with the approach used in biopharmaceutical drug development. Through these examples, it is evident that there is a convergence of thought and an interplay between the pharmaceutical and biopharmaceutical drug development genres, with each perspective benefitting the other.

References

1. OVERVIEW OF BIOPHARMACEUTICALS AND COMPARISON WITH SMALL-MOLECULE DRUG DEVELOPMENT

I. DEVELOPMENT OF BIOPHARMACEUTICALS DEFINED AS NOVEL BIOLOGICS


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I. Development of Biopharmaceuticals Defined as Novel Biologics

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