22 Disposition of Biological Therapeutics: Monoclonal Antibodies, Proteins, and Peptides

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22.1 INTRODUCTION

The past decade has witnessed a major shift in the relative distribution of therapeutic agents being considered to treat human illness. This change in therapeutic landscape has been the result of an overall decrease in the number of traditional or small-molecule drugs being approved, with a concomitant increase in the approval of novel therapeutic biologics or large-molecule drugs. This marked growth of biopharmaceuticals is a reflection of the maturation of biotechnology, both in terms of discovery and development of large-molecule drugs as well as the ability to consistently manufacture these complex molecules. However, despite an impressive evolution of the science, a sound understanding of the factors that control the systemic disposition and pharmacokinetics (PK) of these molecules remains to be defined.

In general terms, a biopharmaceutical is a protein- or nucleic-acid-based pharmaceutical substance used for therapeutic purposes, which is produced by means other than direct extraction from a native (nonengineered) biological source. An example of the latter is seen with insulin, which was extracted from pancreatic tissue of slaughterhouse animals [1]. A high level definition of biopharmaceuticals may be regarded as

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molecules that are produced using recombinant DNA technology in expression cells that are amplified in bacterial or mammalian cell culture.

In addition to the dramatic differences in manufacturing processes between biopharmaceutical drugs and traditional drugs, molecular size is another obvious distinction: the molecules of a biological medicine are much larger, have far more complex spatial structures and are much more diverse, or heterogeneous, than the small molecules that make up traditional drugs. A molecule of a biological medicine is typically a protein made of a chain of 20 to several hundred amino acids within a complex three-dimensional structure. Examples of biopharmaceuticals include monofunctional antibodies that bind one antigen or target, bifunctional antibodies that bind two targets, antibody fragments, peptides, fusion proteins, plasma-derived proteins, and various soluble receptors or receptor ligands.

A second marked difference between biopharmaceuticals and small molecules is observed in the routes of administration. The route of administration of a biopharmaceuticals is very different from a traditional drug taken as a pill or capsule, and each drug is developed with a unique route of administration. Typically, biopharmaceuticals are given intravenously, subcutaneously, or intramuscularly, as they generally cannot be orally administered due to degradation in the gastrointestinal (GI) tract. However, the oral delivery of biopharmaceuticals is an active area of research [2]. In addition, many biopharmaceuticals are administered in a clinical setting, although recently for some chronic indications (e.g., rheumatoid arthritis), molecules exist that are self-administered subcutaneously with autoinjector devices.

Another aspect in which biopharmaceuticals are very different from small-molecule drugs is observed with the routes of elimination and observed PK. The elimination pathways for small-molecule drugs are governed by hepatic metabolism (e.g., cytochromes P450) and the subsequent half-lives are in the range of minutes or hours [3]. In contrast, the half-lives of biopharmaceuticals, antibody drugs, for example, are in the order of weeks [4]. For small-molecule drugs, many safety problems occur around the time of C_{max}; however, for biopharmaceuticals, the focus is typically around total drug exposure or area under the curve (AUC) instead of C_{max}.

Biopharmaceuticals present several advantages over small organic molecule therapeutics in treating many diseases. Foremost, the selectivity of biologics is unsurpassed, and because of this inherent specificity in biological function, protein therapeutics present minimal disturbances to normal biological functions, which limits the potential for untoward side effects. Biopharmaceuticals can be grossly separated into different categories: peptides, proteins (enzymes), and antibodies. For the purpose of this chapter, peptides and proteins have been combined based on similar clearance mechanisms and strategies used to enhance their PK properties as therapeutic agents. Antibodies are described in a separate category. The overall focus of this chapter is to highlight the factors that govern the disposition and PK of the aforementioned biopharmaceuticals.

Peptide or protein biopharmaceutical agents are often designed to replace a deficient or abnormal protein pathway in patients or introduce a novel biological function in order to circumvent a particular physiological deficiency. Peptides of various size and derivation are used as drugs across a wide range of therapeutic regimens and represent one of the fastest growing classes of new drugs in the arena of drug discovery. Within the literature, the distinction between peptide and protein therapeutics can be somewhat inconsistent; thus, by definition, any polymer consisting of two or more amino acids linked by amide bonds is termed a *peptide*.

The increasing importance of peptide therapeutics in drug discovery today can be attributed to two primary factors. First, the evolution of drug discovery techniques, such as phage display and combinatorial chemistry, has lead to the identification of numerous peptide drug candidates that may provide hope as biopharmaceutical agents [5,6]. Second, advances in genetic engineering and chemical synthesis have enabled the large-scale production of peptides that previously were only available in small quantities [7]. Despite the technological advances detailed above, however, when compared with antibodies and proteins, the disposition of peptide drug candidates is plagued with a critical drawback; namely, they are rapidly eliminated from the systemic circulation.

Oral absorption of peptide and protein therapeutics is prohibited by numerous properties associated with the GI tract, including the acidic environment of the stomach (pH 1–3), which is destabilizing to most peptides and proteins, and the production of large amounts of proteolytic enzymes that are secreted into the digestive tract (discussed in detail below). This barrier to the oral delivery of peptides and proteins has required the pursuit of alternate, more invasive routes of administration, including intravenous and subcutaneous dosing. The frequency of using these alternate routes can be minimized with biologic therapeutics that have sufficiently prolonged half-lives (>25 days), which should promote less frequent dosing. However, peptides and proteins are typically cleared from the bloodstream within minutes to hours after administration, and as a result in many cases, there is insufficient drug exposure in the target tissue to elicit a pharmacological effect. The short plasma half-lives observed for peptides typically result from rapid renal clearance due to the small size of the peptides (<60 kDa for the kidney filtration cutoff threshold) and/or from enzymatic digestion in the blood, kidneys, or liver [8,9]. To remedy this issue, a number of strategies have been developed to reduce renal excretion. Consequently, strategies employed to increase the half-life typically require mechanisms to reduce amide hydrolysis or degradation in order to maintain biological activity of the chimeric peptide or protein once serum residence times have been extended.

22.2 RENAL CLEARANCE OF PEPTIDES AND PROTEINS

As described above, one of the major problems for therapeutic peptides is that these drug candidates are rapidly cleared from the circulation via the kidneys [10]. To address the issue of rapid renal elimination, numerous technologies have been developed to increase the *in vivo* plasma residence time of peptide drugs and these will be discussed in more detail in the subsequent sections of this chapter. Overall, these approaches are all designed to make the peptide larger (generally >60 kDa) to retard excretion through the kidneys.

22.3 ENZYMATIC CLEARANCE MECHANISMS OF PEPTIDES AND PROTEINS

Despite the fact that the molecular weight for most peptides is typically below 10 kDa and therefore they should be readily filtered by the glomerulus in the kidney, rapid elimination of peptides due to renal clearance cannot completely account for the exposure

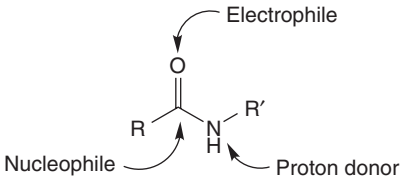

Figure 22.1 Chemical interactions and reactions of the peptide backbone.

issues observed for some peptides, as for example, some half-lives exceed the glomerular filtration rate (GFR) of about 100 mL/min for a normal 70 kg man [11]. To this end, the short half-lives of many small peptides (<10 min) cannot be completely explained by renal clearance and is most likely due to rapid enzymatic degradation.

The process of this metabolic degradation is also called *proteolysis* and is best described in terms of chemical hydrolysis (Fig. 22.1). For peptides, blood, liver, and kidney are probably the most important sites of metabolism, as they contain a wide variety of peptidases [9]. However, because of the physical–chemical properties (e.g., hydrophilicity) of many peptide drugs, proteolytic enzymes in the blood may play a more important role in their metabolism [12].

The mechanism for peptide hydrolysis, like all enzyme-mediated catalytic reactions, requires interactions with appropriate active site groups (Fig. 22.2). For the hydrolysis reaction to occur, the combined action of the active site catalytic groups is essential [13,14], as the peptide bond represents a strong linkage that has a high degree of double-bond character (a consequence of the degree of delocalization of the nitrogen lone pair into the carbonyl group). The strength of the peptide bond weakens in the first step, when a nucleophile, such as water or a serine OH group, attacks the carbonyl carbon atom, which results in a tetrahedral intermediate bearing an oxyanion. In this sequence, the nucleophilic attack is aided by a general base that accepts the proton from the nucleophilic OH group (Fig. 22.2a). The intermediate is stabilized by electrophilic catalysis, which is provided by hydrogen bonds from the oxyanion-binding site to the carbonyl oxygen. This is followed by the expulsion of the leaving group, in this case the amine, from the tetrahedral intermediate. Because the amine is a poor leaving group, it must be protonated by a general acid to be able to depart (Fig. 22.2b). In short, similar to chemical hydrolysis reactions, proteolysis of the peptide bond cleavage proceeds via the formation and decomposition of the tetrahedral intermediate, which is facilitated by general acid–general base catalysis, mediated by a single proton carrier, often an active site histidine side chain in many cases [15].

22.3.1 Mechanism of Peptide Hydrolysis

In general terms, there are four distinct peptidase mechanisms, which are loosely linked to the primary means of the hydrolysis reaction (e.g., serine, cysteine, aspartic, and metallopeptidases). The most obvious difference between the enzyme mechanisms is the presence or absence of a covalent acyl-enzyme intermediate in the reaction pathway [16–18]. The catalyses of serine and cysteine peptidases involve the covalent intermediate (ester and thiolester, respectively) (Fig. 22.2a), while the aspartic and the metallopeptidase catalyses do not (Fig. 22.2b). For hydrolysis reactions carried out by

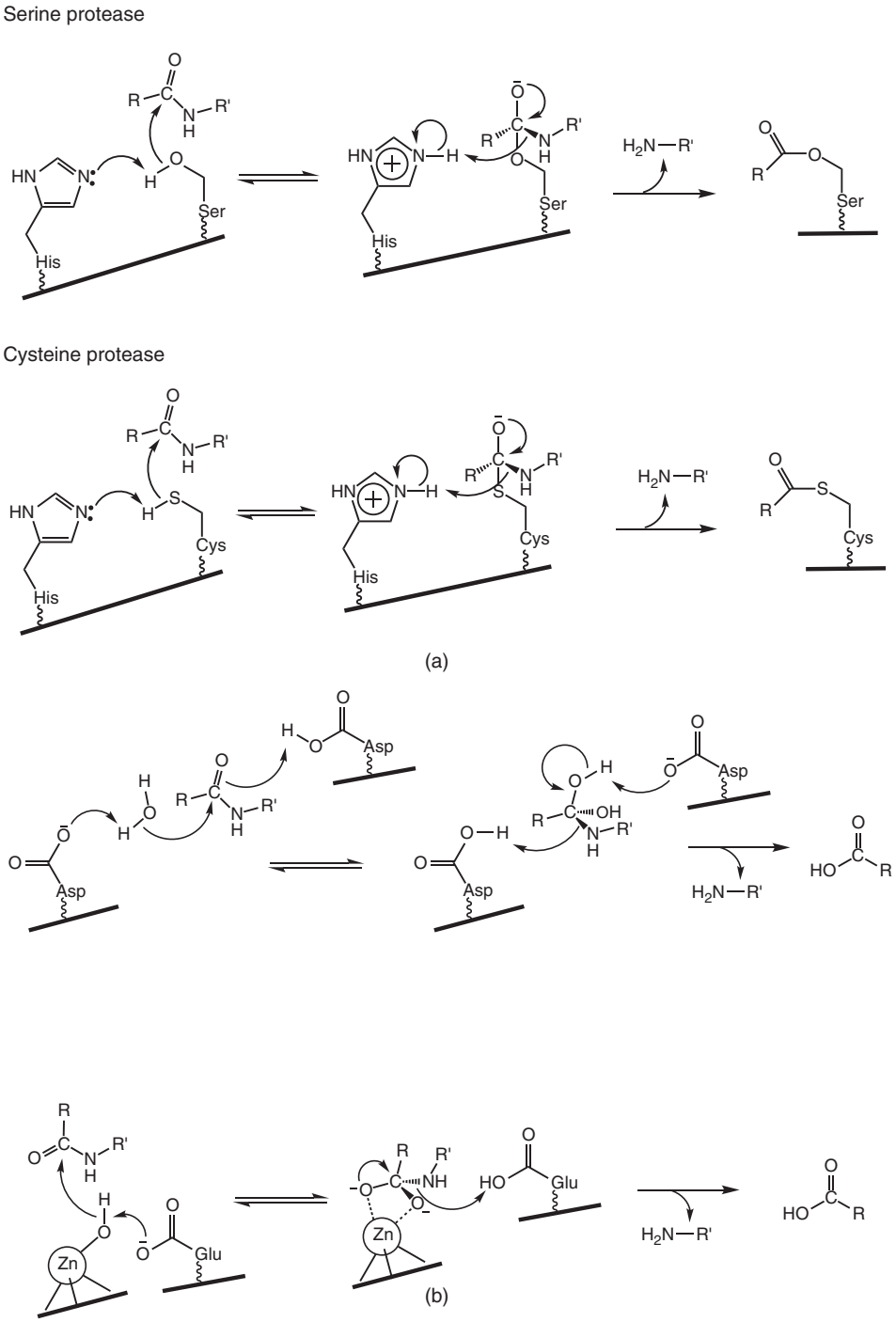

Figure 22.2 (a) Active-site nucleophiles present in serine and cysteine proteases that from an acyl tetrahedral intermediate. (b) Active-site waters acting as nucleophile in metalloproteases.

the latter two groups, the substrate is attacked directly by a water molecule rather than by a serine or cysteine residue.

Interestingly, despite the mechanistic differences, the basic catalytic features of the enzymes described above is quite similar. Hydrolysis of the peptide bond is an addition–elimination reaction involving a tetrahedral intermediate. For these reactions, metal ions and functional organic groups promote hydrolytic cleavage peptides through (i) electrostatic activation of the substrate in the ground state or stabilization of the transition state by metal ion coordination, hydrogen bonding, or proton transfer; (ii) stabilization of leaving group by metal coordination, hydrogen bonding, or proton transfer; (iii) nucleophilic attack on the substrate by functional group or by metal-coordinated hydroxide, which is generated at neutral pH by Lewis-acid activation of metal-coordinated water; and (iv) generation of the nucleophile via proton abstraction by a basic group.

22.3.2 Peptidase Selectivity

Peptide-metabolizing enzymes can be separated into two major classes (Fig. 22.3): the endopeptidases, which break peptide bonds within the molecule of peptides, and the exopeptidases, which catalyze the removal of one or two amino acids from the N-terminus or the C-terminus of a peptide [9]. Exopeptidases that remove dipeptides from the N-terminus of peptides are called *dipeptidyl peptidases*, while those that remove dipeptides from the C-terminus are termed *peptidyl dipeptidases* [19]. In terms of substrate recognition, each peptidase appears to have its own cleavage specificity of peptides. In some cases, multiple enzymes may hydrolyze a particular peptide to

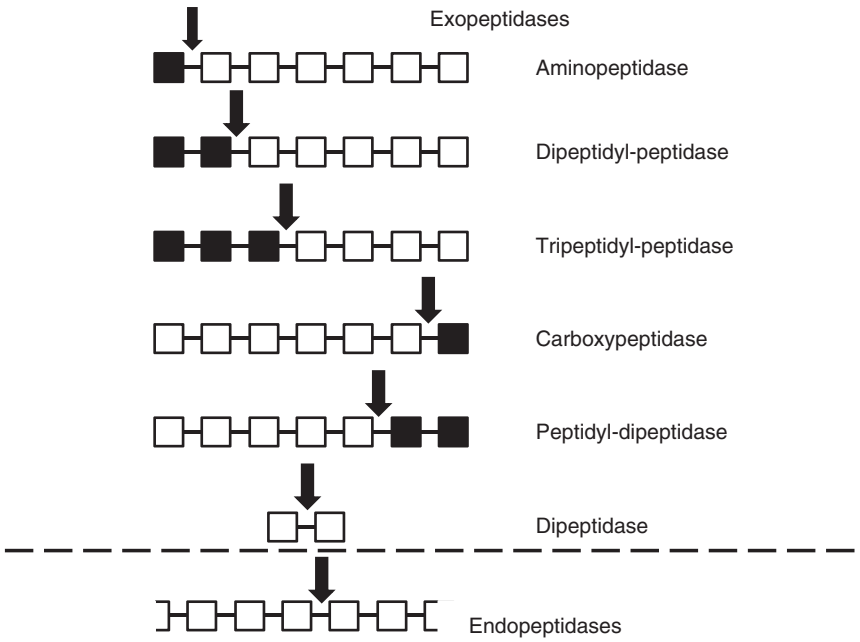

Figure 22.3 An illustration of the two types of peptidases and their general activity.

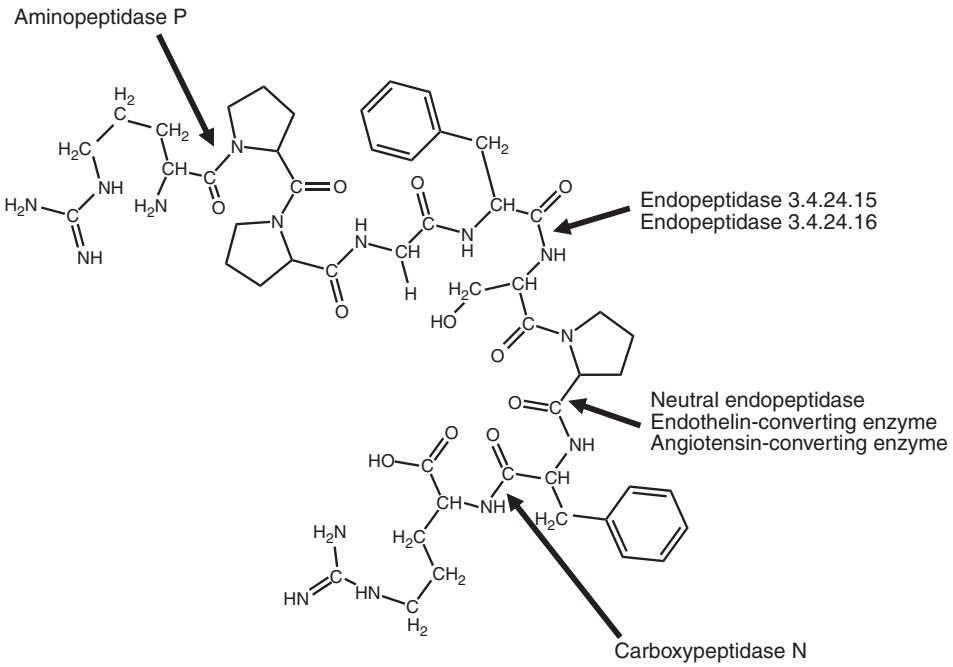

Figure 22.4 Peptidase activity on bradykinin.

yield several products. For example, during a single passage through the pulmonary vascular bed of the lung, no fewer than five of the eight peptide bonds of bradykinin are hydrolyzed (Fig. 22.4) by a variety of peptidases [20]. In contrast, glucagon-like peptide 1 (GLP-1) is specifically hydrolyzed by removal of His-Ala dipeptide of the N-terminus by dipeptidyl peptidase IV (DPPIV) [21]. In this fashion, the information of cleavage specificity of a given peptide drug is important, because knowing the cleavage site allows a rational modification of the peptide drug in order to improve its metabolic stability.

22.4 REDUCING PEPTIDE AND PROTEIN CATABOLISM

22.4.1 Blocking Proteolysis of Peptides and Proteins to Increase Plasma Half-Life

The attachment of polyethyleneglycol (PEG) to a drug molecule, termed *PEGylation*, was first pioneered by Davis and colleagues in the 1970s [7]. The primary objective of this chemical modification was to modify the PK profile of a peptide drug by extending the half-life of the molecule on systemic administration. PEGs are amphiphilic and relatively chemically inert polymers consisting of repetitive units of ethylene oxide [8]. PEGs are named based on the number of ethylene oxide units in the polymer chain; a large variety of PEG molecules with different molecular weights are commercially available. PEGs can be presented with various configurations categorized in two main

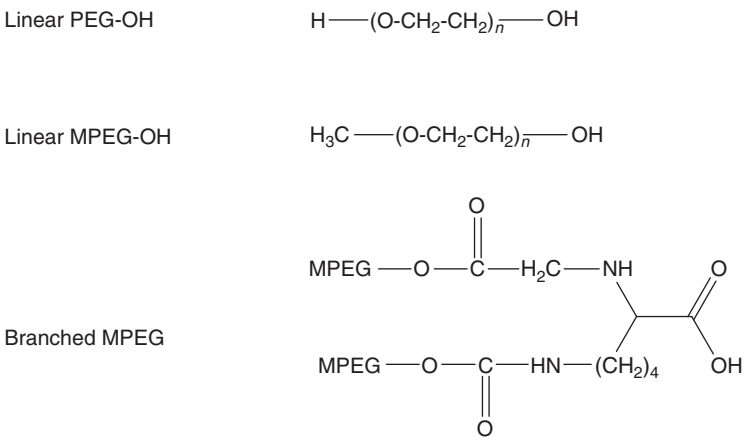

Figure 22.5 Types of pegylation for protein modification.

classes of linear or branched polymers (Fig. 22.5). Moreover, PEGs can be further divided into two groups: PEGs with free hydroxyl groups at both ends and PEGs with one or two methoxylated end group(s) [9]. Multiple low molecular weight PEG molecules (5 kDa), or single high molecular weight PEG molecules (40 kDa) can often be attached at the N-terminus or C-terminus of a peptide without affecting biological activity [10].

The attachment of PEG to peptides is a widely accepted method to increase serum residence times [11]. For example, PHA-794428 is a PEGylated version of somatropin (human growth hormone). The PK of PHA-794428 somatropin is mediated by two mechanisms. The primary mechanism is via clearance by glomerular filtration [12]. When the PK of both molecules (PHA-794428 and somatropin) were evaluated in humans following single subcutaneous administration it was observed that the PEGylation of somatropin with a 40 kDa PEG resulted in a 10- to 20-fold increase in AUC with a similar increase in half-life when compared with somatropin [13].

Site-specific PEGylation has also been achieved in combination with the application of protein chemistry. For example, GLP-1-(7-36) is an endogenous hormone with potential as an antidiabetic agent but due to its extremely short half-life has limited therapeutic potential as the native peptide. Through PEGylation of lysine 34, the residue directly adjacent to the DPPIV peptidase cleavage site, the stability of GLP-1 in the presence of DPPIV is increased >50-fold [22]. The *in vivo* half-life is extended as well but remains short (<1 h) likely due to the renal clearance component of the small PEGylated peptide. Site-specific PEGylation also demonstrated the ability to significantly increase the half-life of IL-15 from minutes to days [23]. In addition, the authors suggest that the strategic placement of the PEG can be useful in the modulation of protein–protein interactions. Several strategies have been developed to introduce PEG molecules specifically into proteins.

The introduction of PEG can also reduce the potential for immunogenicity of some proteins or specific protein sequences. Although site-specific conjugation strategies have been developed, which improve the yield and specificity of the conjugation site, issues remain with the cost and intrinsic heterogeneity of the PEG itself. PEGylated

proteins can also give rise to toxicity. Specifically, renal tubular vacuolation has been observed in animal models. Renally cleared PEGylated proteins or their metabolites may accumulate in the kidney, causing formation of PEG hydrates that interfere with normal glomerular filtration.

The replacement of an L-amino acid by a D-amino acid of a peptide to reduce its susceptibility to enzymatic cleavage is a common strategy to prolong intrinsic plasma half-life of the peptide [24]. For example, natural gonadotropin-releasing hormone (GnRH) has a very short half-life in plasma, which limits its clinical usefulness [25]. However, when a glycine was substituted with a D-tryptophan at position 6 of the natural GnRH, the resulting molecule, triptorelin, has a half-life about 10-fold longer (2 h) than that of natural GnRH, which affords a role for the treatment of central precocious puberty in pediatrics [26]. However, the strategy of substitution of L-amino acids with D-amino acids does not always work and care must be taken to introduce the amino acid substitution in a position where proteolysis occurs [27].

A second example where unnatural amino acid linkages have been employed is observed with β-amino acid peptides [28]. In peptides that comprise α-amino acids, the carboxylic acid group and the amino group are bonded to the same carbon center (also known as the α-carbon because it is one atom away from the carboxylate group). In contrast, β-amino acids have the amino group bonded to the β-carbon (Fig. 22.6). The strategy for this modification is that substitution of an α-amino acid located at the hydrolytically liable peptide cleavage site with a β-amino acid would introduce an extra carbon in the peptide backbone and thus confer resistance to proteolytic cleavage (Fig. 22.7) without necessarily abolishing enzyme binding [29].

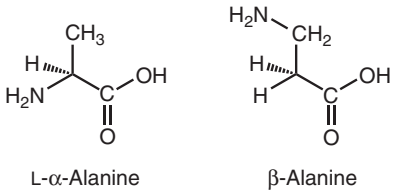

Figure 22.6 Illustration of the natural (L) amino acid compared to non natural (D) amino acid.

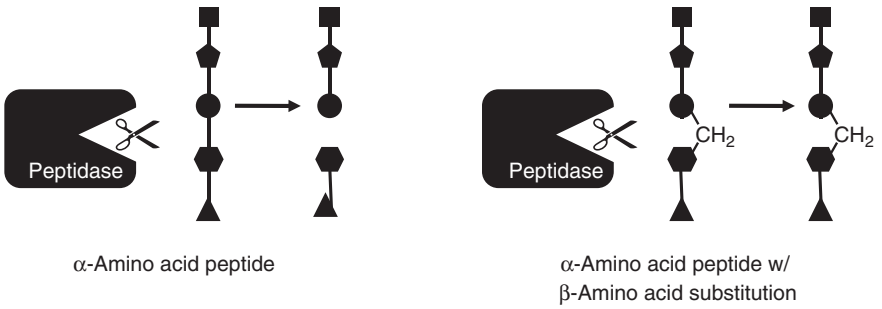

Figure 22.7 Illustration of the natural (L) amino acid compared to non natural (D) amino acid.

As mentioned above, a separate approach to enhance peptide stability is to modify either or both the C-terminus and N-terminus of peptides to minimize their susceptibility to exopeptidases. For instance, N-pyroglutamylation of GLP-1 led to an improved enzymatic stability of and a subsequent increase in *in vivo* glucose-lowering effect in adult obese diabetic (ob/ob) mice [30].

The conjugation of high polymeric mass to peptide drugs has previously been described to decrease the fraction of renal clearance of a peptide and also represents another way to improve their metabolic stability [22,30]. In this fashion, the conjugation of a peptide with PEG increases its hydrodynamic radius and improves its stability toward peptidases. For example a dual-acting peptide's (DAPD) pharmacological effects are attenuated because of its poor metabolic stability. In an effort to overcome a short plasma half-life of the hybrid peptide, DAPD was PEGylated at the C-terminus to enhance its metabolic stability and prolong the duration of pharmacological action [31].

Alternate methods to develop proteolytic resistant peptides have been demonstrated through the cyclization of peptides with olefin metathesis. Specifically, ring-closing metathesis (RCM) links *N*-amino acid to N + 4-amino acid by forming a carbon–carbon bond [32]. The end result is the generation of peptides with increased helical structure and proteolysis resistant peptides that are more suitable as therapeutic candidates due to their increased stability. For example, Fuzeon or enfuvirtide is a 36-amino-acid peptide that confers resistance to HIV-1 infection but remains a last option due to short lived efficacy because of rapid elimination [33]. Through engineering in two ring-closing or stapled sites, the PK of the ring-closed peptide (RCP) showed an ~200-fold increase in half-life from 11 min for the parental peptide to 2040 min [34]. Numerous additional RCP have demonstrated similar increases in stability and proteolysis resistant behavior [35–37]. Alternatively, strategic replacement of amino acids that trigger proteolytic degradation can be selectively replaced with more stable amino acids [38]. Yet another strategy is to replace labile amino acids with the introduction of nonnatural amino acids, which has also demonstrated the ability to increase *in vivo* half-life of peptide and protein fragments.

The primary disadvantage of techniques that focus solely on controlling proteolysis, where a reduction in catabolism can be demonstrated in most of the examples, the principal elimination pathway for the peptide/protein still results in significant renal clearance of the modified peptide/proteins. Future efforts will have to combine proteolytic stabilizing techniques with specific strategies to reduce renal clearance.

22.5 HALF-LIFE EXTENSION OF PEPTIDES AND PROTEINS FUSED TO SERUM PROTEINS

In addition to PEGylation, other approaches toward improving the PK of peptides that are based on binding to or fusion with long-circulating serum proteins such as albumin have been developed. Albumin is the most abundant protein in the blood plasma and is produced in the liver as a monomeric protein of 67 kDa. In addition to its role in regulating the osmotic pressure of plasma, albumin also functions as a transporter of endogenous small molecules such as long-chain fatty acids, bilirubin, and steroid hormones [14]. Albumin also binds with high affinity to a broad range of drugs influencing their PK properties [15].

22.5.1 Attachment to Carrier Proteins

Albumin has a simple molecular structure and is highly stable. It is abundantly present in vascular and extravascular compartments with a circulation half-life of 19 days in humans [16]. Recent studies have shown that this long serum half-life is due to a recycling process mediated by the neonatal Fc receptor (FcRn), similar to that observed for IgG molecules [17]. Some companies have taken advantage of these properties and have fused human serum albumin (HSA) with hormones to serve as a macromolecular carrier for drug delivery [18].

22.5.2 Attachment to Antibody Fragments

A second approach pioneered by Amgen is the attachment of Fc regions to peptides to improve PK. One example is romiplostim, an Fc-fusion protein analog of thrombopoietin, a hormone that regulates platelet production [19]. In this instance, the company made use of the natural antibody constant region half-life extension mechanism, provided by FcRn recycling of antibodies, and any other peptide or protein fused to an antibody Fc domain [20].

22.6 HALF-LIFE EXTENSION OF PEPTIDES AND PROTEINS THROUGH POSTTRANSLATIONAL MODIFICATION

22.6.1 N-Terminal Acetylation

The N-terminus of proteins contains a methionine residue capable of undergoing multiple modifications. With the exposed N-terminus, methionine can be acetylated, triggering methionine-aminopeptidase activity, which cleaves off the acetylated methionine residue. The fate of the newly exposed residue is determined partially by size; specifically, if the amino acid residue is small enough, it too will be acetylated. The acetylations can trigger the ubiquitin degradation system.

22.6.2 Phosphorylation

Phosphorylation of serine or threonine residues has demonstrated the ability to increase clearance of endogenous proteins. For example, the phosphorylation of huntingtin at Ser13 and/or Ser16 leads to accelerated clearance of huntingtin. The phosphorylation also triggers additional modifications, including ubiquitination and acetylation, which has been discussed in further detail later in this chapter. Alternatively, a reduction in autophagy has been observed for Ataxin-7 on posttranslational modification of lysine 257. Blocking anticipated phosphorylation sites may lead to unique biodisposition of protein therapeutics.

22.6.3 Engineered Glycosylation

Protein glycosylation sites also contribute to the diverse biological properties of proteins [39]. Specific changes in glycosylation patterns influence all facets of protein behavior, including expression, proper folding, tertiary stability, biodistribution, activity, and

ultimately the half-life of a protein [40–43]. The ability to affect half-life through changes in the glycosylation state of proteins has been recognized and a significant effort has been made to harness the power of glycosylation in the development of therapeutic proteins [44–46]. The two most common forms of glycosylation are N-linked and O-linked. The general motif for N-linked glycosylation of proteins is Asn-X-Thr/Ser, where X can be any amino acid residue except proline [47]. *N*-Glycans share a common pentasaccharide core and are designated by subcategory as high mannose type, complex type, or hybrid type. In contrast, O-glycosylation does not appear to have a formal consensus sequence and O-linked sugars are commonly attached to serine or threonine residues through an *N*-acetylgalactosamine (GalNAc). Two additional types of glycosylation, *C*-mannosylation and glycophosphatidyl-ionisotol are not discussed further due to limited scope in therapeutic protein design at this time. In addition to the primary sequence motif that indicates potential for glycosylation, it has been proposed that glycosylation sites commonly occur at regions of alternating secondary structure [48]. The increased ability to predict glycosylation sites from primary structure combined with other rule-based computational approaches should aid in the design of novel sites for glycan introduction into proteins in order to enhance the PK properties of protein therapeutics [47].

A number of methods have been employed to alter glycosylation of recombinant proteins. Foremost, the choice of cell line can influence the type and extent of glycosylation, which can further be manipulated by altering expression conditions and media content. Selective pressure of CHO cell lines has been used to isolate mutants in the glycosyltransferase genes [49,50]. Other cell lines are also capable of manufacturing glycoproteins, including human embryonic kidney (HEK) cells, baby hamster kidney (BHK) cells, mouse myeloma cells such as NS0, and the human retinal cell line PERC.6.

In addition to the modification of glycan patterns through strategic choice of expression system, new glycan sites can also be introduced in order to try to improve protein stability, formulation conditions, and PK properties. Site-directed mutagenesis to engineer the glycosylating motif into the therapeutic protein can be attempted [44]. However, this strategy is not trivial and often numerous attempts have to be made in order to introduce novel sites for glycan structures; however, the population of these novel sites to a high degree of fidelity remains quite variable [51]. The variable success of this approach may be indicative of additional factors that contribute to the potential for different protein glycoforms, including microconformations adopted by the protein during biosynthesis and protein folding process. To circumvent these uncertainties, a site-directed mutational approach in combination with specific labeling chemistry has been employed to introduce stoichiometric amounts of glycosylated protein to yield highly homogenous protein preparations [52].

Introducing novel glycan sites may also lead to changes in receptor binding and therefore *in vivo* activity. For example, the specific activity of wild-type DPPIV is significantly higher than that of Gln3191-DPPIV. Alternatively, Gln83 and Gln686 only displayed modest changes in activity compare to wild-type (fully glycosylated enzyme) indicating specific regions of protein glycan structure may have greater impact on function than others [53]. To minimize reduction in activity as a result of introducing novel glycosylation sites internal to the protein, use of the termini of the protein sequence for the introduction of a glycan motif is another strategy that appears more predictive and reproducible [44].

The impact of N-linked glycosylation in native and engineered proteins is readily apparent on review of physical and PK properties of different proteins [54,55]. Another example is follicle-stimulating hormone (FSH), which is a highly glycosylated protein that offers medical advances as a treatment for infertility. The hormone contains two subunits: α and β. Each subunit has two potential sites for glycosylation and structural characterization demonstrated glycol forms with increased sialic acid content tended to possess longer circulation half-lives. Through N-terminal extension of the protein sequence to introduce two novel glycan sites, a modified FSH coined FSH1208 displayed prolonged half-life and efficacy *in vivo* [44]. Lastly, L-asparaginase derived from *Escherichia coli* was chemically modified with *N*-acetylneuraminyl lactose [56]. The thermal stability as well as proteolytic stability was improved for this modified protein. In addition, mouse PK studies were performed with the two materials, which illustrated that the modified protein has a twofold increase in half-life.

22.6.4 Amino Acid Extension Techniques

A polypeptide tethering technique was explored largely based on the premise of the advantages of PEGylation. This strategy focuses on the design of a soluble, chemically stable, and predominantly unstructured polypeptide that provides a maximal hydrodynamic radius to enhance protein PK. The intrinsic physiochemical properties of individual amino acids were carefully explored in designing the proper polypeptide chain; hydrophobic amino acids (e.g., F, I, L, M, V, W, and Y) that typically contribute to compact structures and/or protein aggregation were avoided. Hydrophobic amino acids also play a critical role during binding of peptides to major histocompatibility complex (MHC) class II-driven immune responses. Other amino acids have properties that are undesirable in a molecule intended for clinical usage. For instance, amide-containing side chains such as N, Q, and W are subject to chemical instability during long-term storage of protein drugs, and positively charged side chains such as H, K, and R can facilitate binding to cell membranes. Cysteine residues can cause heterogeneous disulfide cross-linking.

22.7 ANTIBODIES AS THERAPEUTIC PROTEINS

Antibodies, or immunoglobulins, are generated during a humoral immune response critical for the protection of higher organisms. These macromolecules are grouped into five subclasses or isotypes based on the structure of their heavy chains and consist of IgA, IgD, IgE, IgG, and IgM. IgG represents the most important class of antibodies as it is the most abundant in serum at ∼12 mg/mL. The IgG isotype is further divided into four subclasses, IgG1, 2, 3, and 4, with each subclass playing particular roles during an immune response [57]. In general, IgG antibodies have half-lives in human of ∼21 days with the exception of IgG3, which has a 7-day half-life [58], which has been shown to be due to a single amino acid in the Fc region (H435R) which results in less efficient FcRn-dependent recycling (see below [59]).

22.7.1 General Antibody Structure

Each antibody contains two identical heavy and light chains made up of variable and constant regions. Each chain contains an N-terminal antigen-binding variable domain,

or Fab, and a C-terminal constant domain, or Fc. The antigen-binding site is contained within the Fab fragment and is formed by the combination of the heavy and light chain variable region domains, in particular, through three complementary determining regions (CDRs) in each of the heavy and light chain, which define the specificity and affinity of a given antibody for its antigen or target. The light chain constant region has one domain, CL, whereas the heavy chain constant region has three domains, CH1, CH2, and CH3, and a hinge region. Interchain disulfide bonds between CL and CH1 stabilize the association of the heavy and light chain, and multiple interchain disulfide bonds reside in the hinge region between CH1 and CH2 and function to stabilize the heavy chains, while the hinge allows for flexibility of the Fab region relative to the Fc [57,60]. The heavy chain constant domains or Fc region of an immunoglobulin molecule, in particular CH2 and CH3, are important for imparting function onto the molecule; they interact with Fc receptors (FcRs) on numerous effector cells, including macrophages, monocytes, and natural killer cells to potentiate effector functions either through antibody-dependent cellular cytotoxicity (ADCC) or antibody-dependent cellular phagocytosis (ADCP), or with complement proteins to initiate complement-dependent cytotoxicity (CDC) [61,62]. These heavy chain domains also interact with the FcRn (described below) to regulate the functional lifespan of IgG molecules. The general structure of an IgG is shown in Fig. 22.8.

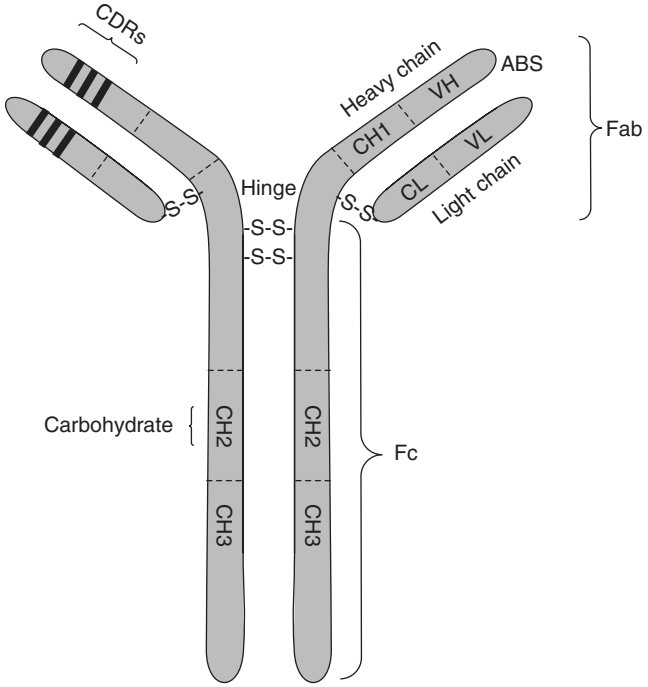

Figure 22.8 General antibody structure. Fc, crystallizable fragment; Fab, antigen-binding fragment; ABS, antigen-binding site; CDR, complimentarity-determining regions; Light chain, VL and CL; Heavy Chain, VH, CH1, CH2, and CH3. The carbohydrate-binding region is located in CH_2 of Fc.

Monoclonal antibodies (mAbs) have become an important therapeutic option for many diseases, including cancer, inflammatory, and infectious diseases (reviewed in Refs 63 and 64). Antibody development platforms include but are not limited to murine hybridomas, chimeric mAbs with human Fc domains, humanized antibodies to remove the mouse/rodent components through molecular biology techniques to reduce immunogenicity, and fully human mAbs derived from phage libraries or transgenic mice that express human IgG loci to generate mAbs [65]. In general, IgG antibody half-life increases with increasing amount of humanization, ranging from mouse to chimeric to humanized to fully human [58,66,67].

22.7.2 Antibody Recycling Through the Neonatal Fc Receptor (FcRn)

FcRn is a heterodimer of the MHC class I such as H-chain and the β-2 microglobulin L-chain [68,69]. The presence of FcRn was first proposed by Brambell *et al*. [70,71], who predicted the presence of a saturable receptor that played a role in pre- and postnatal epithelial transcytosis of IgG across the placenta in humans or intestinal epithelium in rodents, respectively, and in the protection of IgG from catabolism leading to long serum half-life. An additional role for FcRn in immunoglobulin homeostasis was further confirmed in β-2 microglobulin KO [72–74] and FcRn KO mice [75], where IgG isotype Abs were rapidly cleared, as they were present at 10- to 15-fold less concentration than normal and thus lack protection, whereas other isotypes such as IgM and IgA had no change in serum concentration.

The vascular endothelium is the main site where FcRn is expressed [75], but it is also expressed on intestinal, kidney, and bronchial epithelial cells [76] as well as hepatocytes and phagocytic cells of the reticuloendothelial system (RES) and plays a role in clearance and recycling [66].

More recent studies have confirmed a role in immunoglobulin homeostasis by regulating the turnover of IgG [75,77]. Interaction of IgG isotypes with FcRn, particularly IgG1, 2, and 4, occurs after pinocytosis of immunoglobulin molecules from the bloodstream into intracellular endosomes, where, in a pH-dependent manner, antibodies now bind to FcRn and are protected from degradation; those that fail to bind FcRn are catabolized in the lysosomes. On recycling to the cell surface, the IgG molecule is released in the pH 7.4 environment of the bloodstream, thus extending the half-life of the molecule. The proportion of IgG processed through the recycling versus destructive pathway is believed to be important in determining the persistence of IgG in the circulation [78].

There is growing evidence that increasing affinity for FcRn can positively influence the half-life of IgG molecules. By altering Fc-region amino acids that interact with FcRn to increase the endosomal binding at pH 6 and promote recycling, while maintaining release into the bloodstream at pH 7.4, it is this differential pH-dependent binding that has been targeted through Fc engineering approaches to generate mAbs with extended half-lives [62,79]. Extending therapeutic antibody half-life would be advantageous to allow for reduced dosing frequency, decreased dosing amount, increased bioavailability, and improved patient compliance. Much effort has been expended to map the FcRn-binding sites within the IgG constant regions, and the majority of the amino acid residues described as affecting binding have been found to lie within the CH2–CH3 interface of the constant region [59,80] as this region interacts directly with FcRn. The pH dependency of binding is due minimally to conserved histidine residues at positions H310 and H435, which are protonated at acidic and neutral pH or deprotonated at physiological (~7.2–7.4) pH [81,82].

To date several groups have generated Fc region mutants that demonstrate increased half-life in preclinical animal species, including mice and nonhuman primates. Dall'Acqua and colleagues have described the "YTE" mutant after phage display mutagenesis of Fc yielded several sequence variants that improved *in vitro* binding to mouse FcRn at acidic pH and also at pH 7.4 [83]. The residues of interest for the YTE site-directed mutational variant mapped to the CH2 domain, specifically to residues M252(Y), S254(T), and T256(E). PK studies in mice demonstrated those variants that bound more tightly to mouse FcRn at both pH 6 and 7.4, including the YTE variant, and PK profiles with lower exposures and reduced half-lives compared to the parental antibody. The YTE mutations were engineered into the anti-respiratory syncytial virus (RSV) mAb MEDI-524 and yielded improved human FcRn *in vitro* binding at pH 6 but not at pH 7.4. The resulting PK profile of this variant in cynomolgus monkeys demonstrated a fourfold improvement in half-life compared to the parental molecule [84]. This variant, MEDI-557, is currently in phase I clinical trials where validation of improved half-life could be demonstrated.

Using molecular modeling to identify Fc residues that might interact with FcRn, Hinton *et al*. [85,86] demonstrated extended mAb PK profiles by incorporating T250Q/M428L mutations into an IgG2 and IgG1 anti-hepatitis B virus mAb and showed a 2- to 2.5-fold improved half-life in rhesus monkeys, respectively. These variants demonstrated an $\sim 28\times$ and $37\times$ higher affinity for rhesus FcRn binding by surface plasmon resonance at pH 6 versus the parental mAb and showed no binding at pH 7.4 and above for the IgG2 and IgG1 variants. Assuringly, there was little to no negative effect on ADCC or CDC activity, or loss of protein A binding (i.e., a matrix used for purification of monoclonal antibodies) by the variant antibodies, which suggests that the CH2–CH3 interface important for all these interactions is not affected by the incorporation of the mutations.

In the first of several reports to look at the importance of altering Fc region residue N434, Petkova *et al*. [87] generated variants N434A and T307A/E380A/N434A that demonstrated enhanced pH-dependent binding to human FcRn *in vitro* and a correlative half-life improvement *in vivo* versus the parental antibody in mice transgenic for the expression of human FcRn. However, these variants showed no improved half-life in wild-type mice, a result that demonstrates the importance of screening modified Fc variants in the correct molecular context [88].

A second analysis of position N434A by Yeung *et al*. [89] revealed an increased binding to human and primate FcRn at pH 6.0 but negligible binding at pH 7.4 by this variant, which led to an increased exposure and half-life with decreased clearance compared to the wild-type parental mAb. Interestingly, variant N434W, which had 80-fold improved binding to human and primate FcRn at pH 6 as well as increased binding at pH 7.4 revealed a PK profile similar to the wild-type mAb, which demonstrates the importance of preserving the efficient release of IgG at pH 7.4 from FcRn into the bloodstream [89]. This is the first group to demonstrate a potential ceiling for the amount of improvement in differential binding to FcRn in a nonhuman primate system.

Finally, Deng *et al*. demonstrated differential PK with an anti-tumor necrosis factor (TNF) variant mAb with changes in the Fc region of N434A and N434H [90]. The N434A variant mAb had similar *in vitro* binding properties to FcRn as the parental mAb and thus in mice had a similar PK profile, whereas N434H had increased pH 7.4 FcRn binding *in vitro* and demonstrated a more rapid clearance and lower bioavailability versus the parental mAb *in vivo*. Furthermore, both variants demonstrated higher affinity

in vitro binding at pH 6 compared to the parental mAb and demonstrated improved PK characteristics in cynomolgus monkeys, including higher exposures *in vivo*, again confirming the importance of tuning the differential binding effect to promote improved pH 6 binding, while maintaining low binding affinity to promote efficient release at pH 7.4.

In addition to improved PK profiles through FcRn modifications, recent examples of improved pharmacodynamics due to improved FcRn binding have also been reported. Zalevsky *et al*. [91] demonstrated three- to fivefold improved half-lives with the anti-vascular endothelial growth factor (VEGF) mAb bevacizumab and the anti-epidermal growth factor receptor (EGFR) mAb cetuximab with the LS series of mutations (M428L/N434S) in cynomolgus monkeys and huFcRn Tg mice, respectively, and both demonstrated an improved *in vivo* efficacy in xenograft tumor models, the first demonstration of improved pharmacology manifested as increased tumor-suppressing pharmacodynamics due to antibodies with longer half-lives.

Yeung *et al*. from Genentech also demonstrated an improvement of PK in cynomolgus monkeys of bevacizumab variants that contained modified Fc residues. Interesting, the variant T307Q/N434A also demonstrated improved pharmacology in mouse colorectal tumor xenograft models despite having no improved pH-dependent murine FcRn binding or improved PK parameters in mouse [92]. With a finding similar to Deng *et al*. [90], Yeung *et al*. also demonstrated that a 10-fold improvement in pH 6 binding to FcRn may be the upper limit as a greater than 10× increase in binding affinity did not lead to improved PK or half-life but instead led to some degree of a reduced or deleterious effect on PK.

While it appears that there is a direct correlation between improved *in vitro* binding to FcRn at pH 6 and improved *in vivo* PK profiles from the examples presented so far, other Fc-region modification examples that improve FcRn binding *in vitro* have not shown such enhancement *in vivo*. For example, incorporation of the half-life improving T250Q/M428L mutations described by Hinton into an anti-TNF antibody demonstrated ~40-fold improved *in vitro* binding to cynomolgus FcRn but showed no improved PK in cynomolgus monkeys [93,94]. Likewise, Gurbaxani *et al*. have shown no correlation between mAb Fc:FcRn affinity and *in vivo* half-life improvement [95]. Thus, other factors in addition to Fc:FcRn affinity are involved in improvement of PK parameters, which could include absolute (vs relative) FcRn:IgG Fc affinity at both pH 6 and 7.4, the kinetics of the interactions, and the contribution of other clearance mechanisms, including target-mediated disposition, in addition to the FcRn-mediated clearance pathway [79,90] Interestingly, in a recent report, Suzuki *et al*. [96] analyzed the ~25 FDA, EU, and Japanese approved therapeutic antibody and five Fc-fusion protein drugs and demonstrated differential FcRn binding due to affinity differences between Fc interactions with FcRn, which suggests that the overall CH2–CH3 Fc structure may contribute to the relatively rapid clearance of Fc-fusion proteins when compared to full antibodies. Ultimately, clinical validation of several of these modifications or of one modification in several applications will be needed to understand the global utility of this Fc-modified half-life extension approach.

22.7.3 Antibody Targeting and Influence on Pharmacokinetics

The characteristics of an antigen targeted by a monoclonal antibody may play a role in the disposition and elimination of the dosed antibody. In general, anticytokine or antisoluble protein antibodies do not seem to be markedly altered in clearance due to their interaction with their target as evidenced by dose proportional linear clearance.

Cell surface receptors on the other hand may have a significant impact on clearance particularly for mAbs that are cleared through internalization of an antibody–antigen complex [66,97,98]. For example, cetuximab, and trastuzumab, an anti-human epidermal growth factor receptor 2 (HEGFR2) mAb, both demonstrate nonlinear clearance [97,98]. The extent that a membrane-associated antigen affects mAb clearance depends on the density and distribution of the target antigen on the cell surface and the relative internalization efficiency and turnover rate of the receptor [66].

Other influences on mAb PK include the generation of antidrug antibodies that may act to neutralize the drug or increase its clearance, thus reducing its efficacy. The majority of the currently marketed therapeutic proteins and mAbs demonstrate some level of immunogenicity, although use of humanization technologies and fully human antibodies [63] or engineering efforts to remove immunogenic epitopes [99] has reduced the incidence compared to that observed for first-generation mouse mAbs or next-generation chimeric mAbs. Fully human mAbs theoretically are less immunogenic but still contain CDRs that are unique to each therapeutic protein. In general, the fully human proteins or mAbs have demonstrated lower or no degrees of immunogenicity.

22.8 SUMMARY

As the number of approved biologics increases over the coming years, numerous opportunities for the clinical development of novel protein and antibody drugs will occur. Increasing and expanding the understanding of the critical PK and pharmacodynamic parameters will be necessary in order to speed development and to continue to deliver new biological therapeutic agents.

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