PROTEIN CHIMERIZATION: A NEW FRONTIER FOR ENGINEERING PROTEIN THERAPEUTICS WITH IMPROVED PHARMACOKINETICS

A. R. Satvik Iyengar, Shreya Gupta, Snehal Jawalekar, Abhay H. Pande

Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar (Mohali)-160062, Punjab, India (A.R.S.I., S.G., S.J., A.H.P.)

Current address: School of Biomedical Sciences and Pharmacy, Faculty of Health and Medicine, The University of Newcastle, Callaghan, NSW 2308, Australia (Current address for A.R.S.I.)

JPET Fast Forward. Published on April 22, 2019 as DOI: 10.1124/jpet.119.257063
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Improving pharmacokinetics of therapeutic proteins by chimerization

[*]**To whom correspondence should be addressed**: Abhay H. Pande, Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar, (Mohali) -160 062, Punjab, India. Tel.: +91 172 2214 682, Fax: +91 172 2214 692. E-mail: apande@niper.ac.in ; abbupande@yahoo.co.in

Number of text pages: 21

Number of figures: 3

Number of tables: 2

Number of References: 190

Number of Words:

Abstract: 110 words

Introduction: 410 words

Conclusion and future prospects: 336 words

Abbreviations: ABD, Albumin binding domain; CTP, Carboxy terminal peptide, dAB, domain antibody; ELP, Elastin-like peptide; FcRn, neonatal Fc receptor; GLK, gelatin-like protein; HAP, homo amino-acid polymer; HLEP, half-life extension partner; PEG, polyethylene glycol; scFv, single-chain variable fragment; Tf, Transferrin.

Special section: Drug Delivery Technologies

ABSTRACT

With the advancement of medicine, the utility of proteins therapeutic is increasing exponentially. However, a significant number of protein therapeutics suffer from grave limitations, which includes their subpar pharmacokinetics. Here, we have reviewed the emerging field of protein chimerization for improving the short circulatory half-life of protein therapeutics. We have discussed various aspects of protein therapeutics aiming at their mechanism of clearance, and various approaches utilized to increase their short circulatory half-life with principle focus on the concept of chimerization. Furthermore, we have comprehensively reviewed various components of chimera, such as half-life extension partners and linkers; their shortcomings, and prospective work to be undertaken for developing effective chimeric protein therapeutics.

Introduction

With the advent of 21st century and the growth of recombinant DNA technology, there has been a significant progress in the field of biopharmaceuticals, most of which could be attributed to the development in protein therapeutics. In addition, the development of cloning methods and systems for recombinant protein production have certainly advanced the research and development of protein therapeutics (Gellissen, 2005). Recently, it has been reported that 129 distinct biopharmaceuticals have entered the market between Jan 2014 to July 2018 in the US and EU regions (Walsh, 2018). The approval of Humulin™ in 1982 has been a pioneering landmark in journey of recombinant protein therapeutics (De Meyts, 2017). Some other major approvals that followed the approval Humulin™, are Nutropin™ (somatropin) in 1985, Alteplase™ (tissue plasminogen activator) in 1987, followed by Epogen™ (erythropoietin) and Neupogen™ (granulocyte colony stimulating factor) in 1989 and 1991 respectively (De Meyts, 2017). Since the achievements of these milestones, protein therapeutics have come a long way and have proved to be extremely effective for treatment of various diseases (Leader et al., 2008; Lagassé et al., 2017; Walsh, 2014, 2018). It is estimated that at the global level, the share of biopharmaceuticals entities amongst clinically studied pharmaceutical products is around forty percent (Walsh, 2018).

In comparison to the small molecule drugs, the use of protein therapeutics offer substantial advantages, which include lower propensity of adverse events, better tolerance and the wide ranging application of protein therapeutics as a replacement therapy for a variety of disorders (Leader et al., 2008; Walsh, 2010, 2014, 2018). In addition, since protein therapeutics offer shorter timeline of approval from various drug regulatory agencies and better patent protection, this makes them commercially lucrative for the pharmaceutical enterprises (Leader et al., 2008).

However, the application of protein therapeutics still suffers from serious limitations that include poor pharmacokinetics and immunogenicity (De Groot and Scott, 2007; Strohl, 2015). Several mechanisms of protein metabolism can contribute to poor pharmacokinetics of protein therapeutics (Kontermann, 2011, 2016; Meibohm, 2012). In addition, immunogenicity of recombinant protein therapeutics is also one of the major challenges as it leads to development of neutralizing antibodies and undesirable immune responses in the patients, thus resulting in reduced efficacy, rapid excretion and multiple adverse effects (Baker et al., 2010; Chirmule et al., 2012; De Groot and Scott, 2007; Kimchi-Sarfaty et al., 2017; Purcell and Lockey, 2008). In this review, we have particularly focussed upon the use of chimerization approach to improve the pharmacokinetics of protein therapeutics.

Pharmacokinetics of Protein Therapeutics

Amongst various pharmacokinetic parameters, half-life, defined as the time required for the drug concentration to be reduced to one-half in the body, is of particular importance, as it is pivotal in determining the dosage frequency of the drug (Meibohm, 2012).

a) Issues with Non-conjugated Protein Therapeutics. As mentioned above, several protein therapeutics show suboptimal pharmacokinetic attributes. For instance, exenatide, which is an incretin mimetic, has a half-life of ~2.4 hrs. Similarly, glucagon like peptide (GLP-1) has a half-life of upto 2 mins, since it is cleaved readily by dipeptidyl peptidase 4 (DPP-4) (Bond et al., 2006; Diao et al., 2013; Strohl 2015). The short circulatory half-life of the protein therapeutic may result in issues like frequent dosing and patient incompliance, thereby making it important to address the metabolic mechanisms of protein therapeutics (Lagassé et al., 2017; Strohl, 2015).

b) Mechanisms of Protein Metabolism and Clearance. A protein, whether endogenous or externally administered, goes through the process of absorption, distribution, metabolism &

elimination in the body (Strohl, 2015). In terms of delivery, oral route is the most convenient, patient compliant and most preferred route for small molecule drugs (Meibohm, 2012). Although, a recent study has demonstrated successful oral delivery of insulin, but owing to gastrointestinal degradation and issues with bioavailability, oral route is generally not suitable for delivery of therapeutic proteins (Abramson et al., 2019; Meibohm, 2012; Tibbits et al., 2016). Interestingly, protein and peptide therapeutics have been delivered in the form of oral inhalations, as inhaled protein therapeutics with molecular weight of upto 40 kDa could achieve significantly high systemic bioavailability (de Kruijf and Ehrhardt, 2017). It is important to note that inhalable versions of insulin, marketed as Exubera® and Afrezza®, have been approved by the US-FDA (de Kruijf and Ehrhardt, 2017). In practice, the parentral route is considered to be most suitable for administration of protein therapeutics (Meibohm, 2012). Hence, intravenous (i.v.), subcutaneous (s.c.) and intramuscular routes (i.m.) are viable options for administration of protein therapeutics (Meibohm, 2012; Strohl, 2015).

After administration, the absorption of therapeutic proteins occurs mostly through the blood vessels and lymphatics (Strohl, 2015; Meibohm, 2012; Meibohm and Braeckman, 2007; Supersaxo et al., 1990). The distribution of proteins is mostly dependent upon their molecular weight, binding to other proteins in the plasma, their overall charge and their extent of lipophilic nature, however, owing to their large size, their distribution is largely confined to the extracellular compartment (Diao and Meibohm, 2013; Meibohm, 2012; Strohl, 2015). The metabolism and elimination of proteins occurs significantly by proteolytic mechanisms (Meibohm, 2012; Strohl, 2015). The renal route of elimination also contributes considerably to the metabolism of proteins and is mostly selective towards molecular size and charge (Meibohm, 2012; Strohl 2015). Proteins with molecular weight less than 60-70 kDa are eliminated relatively swiftly through glomerular filtration and the passage of negatively charged molecules is impeded in comparison to neutral or cationic molecules (Deen et al.,

2001; Meibohm, 2012; Strohl 2015; Tibbits et al., 2016). There is also a substantial contribution of liver to the metabolism of protein and depending on their size, proteins therapeutics may be taken up into the hepatocytes by passive diffusion or via uptake facilitated by carriers or receptors (Meibohm, 2012). The protein therapeutics also undergo receptor-mediated cellular uptake via the target receptor of the protein therapeutics expressed by any cell, by a process known as "target-mediated drug disposition", which can be regarded as stepping-stone in the elimination of protein therapeutic (Mager, 2006; Meibohm, 2012; Tang et al., 2004). In addition, the neonatal Fc receptor (FcRn) plays a pivotal role in the disposition of IgGs, and protein therapeutics fused with a fraction of Ig (e.g., Fc), or albumin, by a process called FcRn-mediated recycling (**Fig. 1**) (Kim et al., 2007; Kontermann, 2011; Meibohm, 2012; Roopenian and Akilesh, 2007; Schmidt, 2013b; Sockolosky and Szoka, 2015; Wang et al., 2008). It has been observed that, FcRn-mediated recycling, owing to differential affinity of Fc region towards FcRn, is responsible for varying circulatory half-lives of IgG subtypes, with IgG1, IgG2 and IgG4 having substantially long half-lives (~18 to 21 days) in comparison to IgG3 (~ 7 days) (Dirks and Meibohm, 2010; Kim et al., 2007; Meibohm, 2012). Thus, various mechanisms of clearance can be targeted for improving the pharmacokinetics of therapeutic proteins.

Strategies for Improving Pharmacokinetics of Protein Therapeutics

To improve the pharmacokinetic attributes of therapeutic proteins several approaches have been devised (**Fig. 2**) (Kontermann, 2011, 2016; Strohl, 2015; Zaman et al., 2019).

a) Mechanisms for Improvement in Pharmacokinetics of Protein Therapeutics. One of the most common approach is to increase the hydrodynamic radius of protein therapeutics, which would lead to reduced renal clearance and increased residence time in the circulation (Kontermann, 2011, 2016; Strohl, 2015). Second approach is to utilize formulations that

entrap the therapeutic proteins thereby resulting in decreased proteolysis and recognition by phagocytic cells (Hartung and Bendas, 2012). A third approach is to impart negative charge onto the proteins, which would selectively impede their renal clearance (Kontermann, 2011, 2016; Meibohm, 2012; Strohl, 2015). Lastly, attachment or genetic fusion with another protein/domain or recombinant polymeric peptide repeats that have inherently long half-life can be used as an approach to increase the hydrodynamic radius and facilitate FcRn-mediated recycling (Kontermann 2016; Meibohm, 2012; Strohl, 2015). These approaches of improving pharmacokinetics of therapeutic proteins are discussed in this section.

b) Conjugation, Attachment and Modification. Researchers have devised several methods of chemical conjugation; with one of the classical method being PEGylation (Kontermann, 2012; Zaman et al, 2019). PEGylation is described as covalent bonding of polyethylene glycol (PEG) moieties with therapeutic proteins (Kontermann, 2012). It has been observed that, binding of a few molecules of water to the ethylene glycol subunit results in up to 10-fold increase in size of the PEGylated molecule in comparison to a protein of similar mass (Swierczewska et al., 2015). Therefore, binding to PEG leads to considerable increase in the size and mass of PEGylated molecule, thereby increasing the hydrodynamic radius and impeding the clearance of PEGylated molecule, consequently resulting in increased half-life (Kontermann, 2012). Furthermore, the PEG conjugation also protects the therapeutic protein from proteolysis and immunological response (Jevševar and Kunstelj, 2012; Swierczewska et al., 2015). In addition, conjugation with other carbohydrates (e.g., glycosylation, HESylation, polysialylation) and synthetic polypeptides (e.g., PEPylation) have also gained attention as an alternative to PEGylation (Fares, 2012; Hou et al., 2019; Li and d'Anjou, 2009; Kontermann 2011, 2012, 2016; Sinclair and Elliott, 2005; Solá and Griebenow, 2010; Vugmeyester et al., 2012).

c) Encapsulation and Surface Binding. Several pharmaceutical delivery systems, such as liposomes and other polymeric formulations, have also been utilized to improve the circulatory half-life of therapeutic proteins (Colletier et al., 2002; Hartung and Bendas, 2012; Landfester et al., 2012). Liposomes are bilayered phospholipid vesicles with hydrophilic interior; moreover, PEG modification of liposomes helps surpass clearance by reticuloendothelial system or by phagocytic cells, thereby leading to increased half-life (Hartung and Bendas, 2012). Encapsulation in liposomes have been shown to increase the half-life of tumor necrosis factor (TNF-α) and interleukin 2 (IL-2) (Hartung and Bendas, 2012). Another approach to improve pharmacokinetics using liposomes, is non-covalent interaction of the protein (for e.g., recombinant factor VIII (rVIII)) on the surface of PEGylated liposomes (Hartung and Bendas, 2012). In addition, certain polymeric nanoparticles, nanocapsules and nanoghosts are also used for pharmacokinetically efficient delivery of peptides and proteins (Kontermann, 2012; Krishnamurthy et al., 2019; Landfester et al., 2012; Pisal et al., 2010; Swed et al., 2014).

d) Focus on Chimerization of Protein Therapeutics.

1) The Concept and Molecular Biology of Chimerization. The word ‘chimaera’ also spelled as chimera, hold its origins in the Greek mythology, where chimaera is defined as a monstrous creature of Lycia, which is believed to be a hybrid (Peck, 1898). Chimaera is portrayed as having combination of physical attributes of a lion, a goat and a snake (Peck, 1898). The concept of chimerism is significant in terms of genetics because it exists in both animal and plant kingdoms, furthermore, experimentally generated chimeras have been serving as useful tools for developmental biologists (Dunsford, 1953; Eckardt et al., 2011; Fontaine-Pérus, 1999; Norris et al., 1983; Santelices, 2004). Moreover, certain chimeras, in their incipient stages, could also act as source of organs for transplantation in the future (Blakemore, 2017).

In terms of protein therapeutics, chimerization is a process where a hybrid is generated with genetic fusion of multiple distinct entities (Baldo, 2015). Hence, in protein chimerization, the idea behind the mythological creature 'chimera' is utilized to produce a molecule with superior properties. A typical protein chimera is produced by connecting genes of the protein molecule of therapeutic interest ('effector') to another protein/domain (referred to as 'helper') with the help of a 'linker', where the effector molecule possesses a myriad of pharmacological activities (Czajkowsky et al., 2012; Baldo, 2015; Schmidt, 2013a). The protein chimera or chimerized protein is also referred to as a 'fusion protein' or 'chimeric protein', and these terms are used interchangeably (Baldo, 2015; Kontermann, 2012, 2016). The 'helper' imparts stability to the molecule and helps in targeting the effector (Baldo, 2015). Several of these helpers utilized as half-life extension partners (HLEP) are either full length proteins or truncated domains of proteins, and result in a considerable increase in the half-lives of the effector molecules (Kontermann, 2011, 2016; Strohl, 2015). The purpose of the 'linker' is to connect the 'effector' and 'helper' in a way that allows optimum functionality so that the whole chimerized molecule can execute its operation (Baldo, 2015, Schmidt, 2013a).

The protein chimerization for half-life extension involves, fusing the therapeutic protein with a HLEP, which has inherently longer half-life for e.g., albumin fusion (**Fig. 3**). (Meibohm, 2012; Strohl, 2015; Sun and Micheals, 2018). Fusing multiple repeats of amino acid sequences (recombinant polymeric peptide repeats) with the effector protein, also lead to increased hydrodynamic radius, for e.g., elastin like polypeptides (ELPs) (Kontermann 2016; Strohl, 2015) (**Fig. 3**). Using a negatively charged protein fragment that decelerates renal elimination has also been utilized as HLEP, for e.g., C-terminal peptide (CTP) of human chorionic gonadotropic hormone (Kontermann 2016; Meibohm, 2012; Strohl, 2015; Sun and Micheals, 2018) (**Fig. 3**).

2) Various HLE Partners Utilized to Enhance Half-Life of Protein Therapeutics. As discussed above, a typical chimeric protein therapeutic is composed of three components; the effector protein is fused to half-life extension partner (HLEP) via a linker peptide. Various partners/helpers utilized for improving the pharmacokinetics of therapeutic effector proteins are elaborated in this section (**Fig. 3**).

i) Crystallisable Fragment of Immunoglobulin (Fc). Fusion with the Fc region of IgG is one of the most popular approach utilized to prolong the half-lives of protein therapeutics (Kontermann, 2016; Richter et al., 2019; Strohl, 2015; Wu and Sun, 2014; Zaman et al., 2019). As discussed above, FcRn-mediated recycling plays an important role in recirculation of proteins, particularly in case of fusions containing the Fc region (**Fig. 1**) (Meibohm, 2012; Rath et al., 2015; Sockolosky and Szoka, 2015; Ward and Ober, 2018). Since the immunoglobulins~~,~~ contain the Fc-region, they have substantially long half-lives for e.g., IgG1, IgG2 and IgG4 (Huang, 2009; Kontermann, 2016; Meibohm, 2012; Sockolosky and Szoka, 2015). In case of therapeutic antibodies, half-life extension of as much as four weeks have been observed (Keizer et al., 2010; Kontermann, 2016). The function of Fc-fusion is to bestow properties such as FcRn-mediated recycling to decrease the metabolism of the therapeutic proteins (Kontermann, 2016; Rath et al., 2015; Strohl 2015). However, Fc fusions do not possess half-lives as long as the immunoglobulins, which could be in part due to the involvement of fragment antigen binding (Fab) of Ig towards FcRn binding (Schoch et al., 2015; Souders et al., 2015; Suzuki et al., 2010; Unverdorben et al., 2016). Several undesirable effects are also associated with Fc fusion, such as, it may facilitate antibody dependent cellular cytotoxicity (ADCC) and phagocytosis and complement fixation (Kontermann, 2016). By introducing mutations at specific positions in the Fc region and using particular isotypes of IgG that do not bear such effects, such as IgG4, these undesirable effects can be subsided (Kontermann, 2016). Fc fusion also provides the flexibility of fusing

effector molecule(s) at either or both *N*- and *C*-terminus (Kontermann, 2016). Several Fc fusions are developed and approved by various regulatory authorities, including etanercept (TNF receptor fusion), aflibercept (VEGF receptor fusion) and rilonacept (IL-1 receptor fusion) (Huang, 2009; Jazayeri and Carroll, 2008; Kontermann, 2016; Strohl, 2015). Mutations in the Fc regions have been performed for optimizing the properties of the resultant fusions and to overcome undesirable effects. For example, dulaglutide, a fusion of GLP-1 with Fc region of IgG4, is a GLP-1 receptor agonist with mutations F234A and L235A (Kontermann, 2016). These mutations decease Fcγ receptors interaction and ADCC induction (Kontermann, 2016). The mutation S228P in dulaglutide prevents the formation of half-antibodies (Glaesner et al., 2010; Kontermann, 2016). When compared to other GLP-1 agonists, such as exenatide and liraglutide, which require daily administration, dulaglutide has to be applied only once in a week (Kontermaan, 2016). Mutations which cause an upsurge in FcRn binding, thereby increasing the half-life, can also be incorporated in the Fc region (Kontermann, 2016; Kuo and Aveson, 2011; Presta, 2008; Wang et al., 2014a). Several mutations that have led to considerable half-life improvements of IgGs are reported in the literature (Bas et al., 2019; Kontermann, 2016; Kuo and Aveson, 2011; Presta, 2008; Wang et al., 2014a). For example, motavizumab which is a monoclonal antibody for respiratory syncytial virus incorporates three mutations in the Fc region namely M252Y, S254T and T256E (Kontermann, 2016; Robbie et al., 2013). These mutations increase the FcRn binding upto 10-fold and the half-life in serum from 2- to 4-fold (Kontermann 2016; Liu et al., 2018; Robbie et al., 2013). Moreover, it has been reported that hypersialylation of aspargine residue at 297 position, achieved through deletion of glutamate residue at 294, could prolong the residence of Fc in the serum (Bas et al., 2019). Also, fusion with certain FcRn binding peptides (FcRnBPs), has also led to enhancement in the half-life of Fab domain (Datta-Mannan et al., 2019). Apart from direct Fc chimerization, fusion with IgG-binding

domain of *streptococcal* G protein, which possesses binding capability towards Fc, has also been utilized for half-life extension (Unverdorben et al., 2015; Zong et al., 2019). Eftrenonacog-α, marketed as Alprolix®, which is a Fc-fused-factor IX for treatment of haemophilia, is an example of recently approved Fc fusion therapeutic **(Table 1)** (Graf, 2018; Shapiro et al., 2012; Shapiro et al., 2019; Strohl, 2015). In addition, Eloctate® (Fc fused factor VIII) has also been studied clinically for the treatment of haemophilia (Mahlangu et al., 2018). Studies with Fc-fused cocaine hydrolase for the treatment of cocaine abuse and fusion of growth hormone with single chain Fc-dimer have also been performed (Chen et al., 2018; Zhou et al., 2017).

ii) Albumin. It has been observed that the half-life of albumin, a predominantly abundant protein in the serum, is upto 19 days (Kontermann, 2016; Sleep, 2015). FcRn-mediated recycling also plays a pivotal role in preventing albumin catabolism, but since albumin binds to FcRn at a site different from IgGs, there is no interference with the recycling of IgGs (Larsen et al., 2018; Sand et al., 2015). The first and the third domain of albumin are found to interact with FcRn in a pH-dependent manner (Sand et al., 2014). Since albumin acts as a transport protein, it can be used for half-life improvement of protein therapeutics (Kontermann, 2016; Sleep, 2015). Half-life extension approaches using albumin involve either binding to albumin, via various interactions and conjugations, fusion of target proteins to albumin-binding moieties or by generating chimeras with direct albumin genetic fusion (Fuchs and Igney, 2017; Kontermann, 2016; Larsen et al., 2016; Lee and Youn, 2016; Ramírez-Andersen et al., 2018; Taraghdari et al., 2019; Zaman et al., 2019). Albumin-binding moieties include certain fragments of antibodies (such as Fab), single chain variable fragment (scFv), domain antibodies (dAbs), nanobodies, albumin-binding domains (ABDs) and DARPin® domains and albumin binding peptides (Goodall et al., 2015; Ikeda et al., 2019; Jacobs et al., 2015; Jank et al., 2019; Khodabakhsh et al., 2018; Kontermann, 2016; Li et al.,

2016; Rycroft and Holt, 2012; Schmidt et al., 2013; Seijsing et al., 2018; Sleep, 2015; Sleep et al., 2013; Steiner et al., 2017; Tijink et al., 2008; Van Roy et al., 2015). When albumin binding moieties, attached to therapeutic proteins by genetic fusion, are administered, they bind to serum albumin thereby increasing the half-life of fused therapeutic (Kontermann, 2016). However, direct fusion with albumin is one of the significant approaches for half-life extension and has been utilized for a wide variety of molecules with therapeutic properties (Hoogenboezem and Duvall 2018; Kontermann, 2016, Müller et al., 2007, Strohl, 2015). One notable example is the fusion of human serum albumin with GLP-1, referred to as albiglutide, has a half-life ranging from 4 to 7 days, and has been approved for diabetes treatment **(Table 1)** (Bush et al., 2009; Trujillo et al., 2014; Strohl, 2015). Another example is the fusion of albumin to factor-IX, referred to as albutrepenonacog alfa and marketed as Idelvion® for haemophilia treatment (Chia et al., 2018; Graf, 2018; Santagostino et al., 2016). Moreover, other clotting factors such as factor VIIa, VIII and X have also been fused with albumin (Ferrarese et al., 2019; Schulte, 2009; Tiede et al., 2015). Recently, fusion of albumin with glucarpidase and Kunitz protease inhibitor domain of protease nexin 2 have also been investigated (AlQahtani et al., 2019; Sheffield et al., 2018). Derivatives of albumin with enhanced properties can be produced by introducing mutations at certain positions. Such as a mutation K573P results in enhanced FcRn affinity by upto 11-fold, thereby leading to increased half-life (Andersen et al., 2014; Kontermann, 2016). In addition, fusion to albumin can be performed at both *N*- or *C*-terminus or individually at either *N*- or *C*- terminus (Andersen et al., 2014; Kontermann, 2016; McDonagh et al., 2012; Müller et al., 2007; Rogers et al., 2015; Strohl, 2015).

iii) Transferrin. Transferrin (Tf), which is a highly abundant monomeric glycoprotein of 80 kDa and takes part in uptake of iron in the cells by exocytosis and receptor-mediated endocytosis (through Tf receptor), is also used as HLEP (Chen et al, 2013a; Kontermann,

2016; Li and Qian, 2002; Strohl, 2015). It is found to have a half-life of upto 17 days, however the glycosylated counterpart of Tf may have a reduced half-life of upto 10 days, and hence the non-glycosylated Tf is preferred as a fusion partner (Kim et al., 2010; Kontermann, 2016; Strohl, 2015). The prominent example of Tf fusion is the fusion of GLP-1 agonist and exendin-4 with non-glycosylated Tf (Kim et al., 2010). Fusions of GLP-1 with Tf have shown a half-life of 44 hrs in cynomolgus monkey model and resulted in decrease in blood glucose and increase in insulin secretion (Kim et al., 2010; Kontermann, 2016). In another study, the Tf-proinsulin fusion displayed a substantial increment in the elimination half-life of 15-folds in comparison to the half-life of unfused proinsulin **(Table 1)** (Wang et al., 2014b). Moreover, a recent study with Tf-proinsulin fusion demonstrates that the fusion possessed a lowering effect on blood glucose levels for upto 40 hrs (Shao et al., 2016). Other examples include development of Tf fusions with growth hormone and granulocyte colony stimulating factor (Chen et al, 2011; Kontermann, 2016).

iv) Fusion with Other Half-Life Extension Partners. As a substitute to PEGylation, certain approaches have been developed which utilize recombinant fusion of target protein with recombinant polymeric peptide repeats that are also termed as PEG mimetics (Kontermann, 2012, 2016; Strohl, 2015; Sun and Micheals, 2018). Fusion to these recombinant polypeptide chains leads to increase in the hydrodynamic radius of the chimeric proteins thereby, causing impeded renal elimination (Kontermann 2016; Strohl, 2015). The most prominent examples to this approach include XTENylation, ELPylation and PASylation (Kontermann 2016; Strohl, 2015; Sun and Micheals, 2018). XTENylation is a half-life extension approach developed by Amunix, which utilizes fusion of polymeric amino acid sequences (alanine, glutamic acid, glycine, proline, serine and threonine), to the target protein (Schellenberger et al., 2009; Strohl, 2015). XTEN polymer of 864 amino acids in length has been shown to extend half-lives of several therapeutically important proteins both in the form of fusion and

chemical conjugation (Alters et al., 2012; Podust et al., 2013, 2016; Schellenberger et al., 2009; Strohl, 2015). One of the examples for XTEN fusions is VRS-317 (somavaratan), a fusion of XTEN and human growth hormone (hGH) **(Table 1)** (Cleland et al., 2012; Moore et al., 2016; Yuen et al., 2013). Another example of polypeptide repeats used for half-life extension is ELPylation, which utilizes peptide polymer repeats that are generally found in hydrophobic domain of Elastin, hence the term, Elastin-like peptides (ELPs) (Hassouneh et al., 2012; MacEwan and Chilkoti, 2014; Strohl, 2015). ELPs are formed with combination of five peptides valine-proline-glycine-x-glycine, where x could be any guest amino acid apart from proline (Floss et al., 2010, 2013; Hassouneh et al., 2012; MacEwan and Chilkoti, 2014; Strohl, 2015). ELPs are biodegradable owing to their metabolism by the elastases in the body (Strohl, 2015). ELPylation impedes kidney clearance by increasing the hydrodynamic radius, thereby enhancing the half-life of protein therapeutics (Conrad et al., 2011). Some examples of ELPylated fusions include Glymera ™ (PB1023) which is a GLP-1 fusion, and Vasomera™, a fusion with vasoactive intenstinal peptide **(Table 1)** (Strohl, 2015). Recently, a study has shown that ELPylation of interferon alpha (IFN-α) led to the formation of sustained release depot that significantly prolonged the action of IFN-α for anti-tumor activity (Wang et al., 2019). PASylation is another approach towards half-life extension of therapeutic proteins, where recombinant polymeric repeats are formed by using proline, alanine and serine amino acids (therefore termed as PASylation) (Binder and Skerra, 2017; Breibeck and Skerra, 2017; Gebauer and Skerra, 2018; Schlapschy et al., 2013). Several studies for half-life extension using PASylation are reported in the literature, one of the notable examples being the PASylation of exenatide where a PAS repeat of 600 amino acids led upto 100-folds increment in the half-life (Harari et al., 2014; Gebauer and Skerra, 2018; Strohl, 2015; Schlapschy et al., 2013). A recent example for PASylation, is the fusion of Adnectin C with PAS repeat of 200 amino acids, which led to increase in half-life by a factor

of 4.5 **(Table 1)** (Aghaabdollahian et al., 2019). In addition to the above-mentioned approaches for half-life extension, some others include HAPylation, which utilizes homo-amino-acid polymers (HAPs) that consist of glycine rich repeat sequences, and Gelatin-like protein (GLK) (Kontermann 2009; Huang et al., 2010; Schlapschy et al., 2007; Strohl, 2015). Apart from using polypeptide repeats, fusions of therapeutic proteins with carboxy terminal peptide (CTP) of the β-subunit of human chorionic gonadotrophic hormone have also been generated (Calo et al, 2015; Fares et al, 1992; Fares and Azzam, 2019). This imparts or increases the negative charge on the chimeric protein thereby impeding renal elimination (Calo et al., 2015; Fares et al., 1992). Some recent examples of CTP fusion include MOD-4023, which is a fusion with human growth hormone and MOD-5014 that is CTP fusion with factor VIIa **(Table 1)** (Bar-Ilan et al., 2018; Hershkovitz et al., 2016; Strasburger et al., 2017).

v) Linkers: the Bond Between the 'Effector' and 'Helper'. The selection or rational design of a linker to join the effector and HLEP protein is a critical area in chimeric protein technology (Chen et al., 2013a, 2013b; Kontermann, 2011, 2016). The linker peptide helps to connect the protein components, and could also have a pivotal role in inter-domain/ inter-protein interactions and in preserving the respective biological activity (Gokhale et al., 2000). Moreover, linkers can also have positive effects on the stability, activity and pharmacokinetics of chimeric proteins (Chen et al., 2013b). Direct fusion of proteins to generate a chimera without any linker may lead to unwanted effects, such as low yield and reduced activity (Amet et al., 2009; Bai et al., 2005; Bai and Shen., 2006; Chen et al., 2013b; Zhao et al., 2008). Thus, choice and design of linkers is very important. Based on their attributes, linkers can be classified as flexible, rigid and cleavable **(Table 2)** (Chen et al., 2013a, 2013b). Flexible linkers are used when the protein partners in the chimera require movement, interaction and maintenance of a certain distance between them. They are

composed of small, non-polar or polar amino acids. The polar amino acids facilitate hydrogen bonding and the small size of amino acids helps to achieve flexibility (Argos et al., 1990; Chen et al., 2013a, 2013b). The most commonly used flexible linkers is the GS linker with sequences consisting of glycine and serine residues and the sequence formula (Gly-Gly-Gly-Gly-Ser)n or $(G_4S)_n$ is the most popular among flexible linkers; where n is the number of repeats (Chen et al., 2013a, 2013b) **(Table 2)**. Other examples of flexible linkers include $(Gly)_8$ or $(G)_8$ linker, and the linkers used to create single-chain variable fragment (scFv) such as KESGSVS and EGKSSGSGSESKST (Bird et al., 1988; Chen et al., 2013a, 2013b; Sabourin et al., 2007). The flexible linkers however, owing to their high flexibility, might not allow the separation of the operational domains/protein partners (Chen et al., 2013b). Moreover, it has been reported that the use of flexible linker may result in failed expression of certain chimeric constructs (Amet et al., 2009; Bai and Shen, 2006; Chen et al., 2013b).

Therefore, where a spatial separation of domains/protein partners is required, rigid linkers are utilized since they act as inflexible spacer peptides that separate domains/protein partners **(Table 2)**. Due to this inflexible distance, the domains/protein partners are relatively free to perform their respective functions (Chen et al., 2013a, 2013b). Two types of rigid linkers are predominantly mentioned in the literature. The first is the α-helical linker with the formula $(EAAAK)_n$, where n is the number of repeats, the second is the rigid $(XP)_n$ linker, where n is number of repeats and P is proline; here X can be any amino acid, however, generally alanine, lysine and glutamine are utilized **(Table 2)** (Chen et al., 2013a, 2013b). Due to the inability of proline to form hydrogen bonds, the domain-linker/protein-linker interaction is avoided, and this increases the rigidity leading to efficient separation of protein partners in the chimera (Chen et al., 2013a, 2013b). The first two categories of linkers contain non-cleavable and stable sequence of peptides that can impart several advantages including conformational flexibility, improved stability and activity (Chen et al., 2013a, 2013b).

However, with the use of these stable peptide linkers several detrimental effects such as decrements in activity, steric hindrances in domains/protein partners have been observed (Chen et al., 2013a, 2013b). To overcome this, a third category, of the cleavable linkers, is used with the intention of releasing free functional domains/protein partners *in vivo* (Chen et al., 2010, 2013a, 2013b) **(Table 2)**. These linkers are cleaved under some specific conditions such as presence of reducing reagents or proteases. The first type in this category is the *in vivo* cleavable disulfide linker. One of the examples of this is the dithiocyclopeptide linker, which is cleaved in presence of a reducing environment (Chen et al., 2013a, 2013b). **(Table 2)**. The second type are the *in vivo* protease sensitive linkers where the chimera is designed with a linker containing a protease sensitive sequence (Chen et al., 2013a, 2013b). An example of this is a fusion between recombinant factor IX (rFIX) and albumin, where a sequence VSQTSKLTRAETVFPDV, from N-terminal region of FIX, which is susceptible to proteolytic cleavage, is incorporated **(Table 2)** (Chen et al., 2013a, 2013b; Schulte et al., 2009). This causes several fold increments in the clotting activity of the chimeric protein as compared to the chimeric protein with non-cleavable linkers (Chen et al., 2013b; Schulte et al., 2009).

Protein Chimerization for Miscellaneous Specialized Purposes

The choice of 'helper' protein is primarily based on the desired functions of the chimeric protein, since the helper partners (apart from half-life extension) can also be utilized to generate chimeras with other properties such as cancer targeting and permeation through blood brain barrier (BBB) (Hoogenboezem and Duvall 2018; Jank et al., 2019; Pardridge, 2015; Tijink et al., 2008). For instance, human paraoxanase-1 (PON-1) fused with the *C*-terminus of heavy chain of monoclonal antibody against human insulin receptor (HIRMAb) has the ability to cross BBB (Boado et al., 2008). In addition, NHS-IL12, a chimera of IL-2 and NHS76 (an IgG1 targeting tumor necrosis) not only displayed half-life extension, but

also showed selectivity in targeting tumors in mice (Fallon et al., 2014). In addition, an antibody developed with fusion of scFv with Fc, showed significant *in vitro* growth inhibition of *Staphylococcus aureus* (Wang et al., 2019**)**.

Chimerization with HLEPs could also have synergistic effect on the pharmacological activity of the effector, for eg., Fc fusions with Osteopontin and Follistatin-288 have shown to enhance the osteogenic effect and promote localized growth of skeletal muscles, respectively (Castonguay et al., 2019; Rattanpasit et al., 2019). In addition, fusion of canine interferon gamma with canine serum albumin not only lead to improved pharmacokinetics but also improved antitumor efficacy (Li et al., 2019). Similarly, fusion of recombinant immunotoxins with ABDs led to increased half-life and significant increment in the antitumor effects of these immunotoxins (Wei et al., 2018). Thus, effector proteins with different functions can be chimerized together to produce a multifunctional protein therapeutic (Jochems et al., 2019).

Comparison and Outlook on Shortcomings of Protein Chimerization versus Other Approaches Utilized for Improvement of Pharmacokinetics

Various approaches of half-life extension have their respective shortcomings. PEGylation for instance has many disadvantages that include reduced activity of the conjugated protein, increased aggregation, unusual behaviour of the therapeutic such as increase in activity in some cases (Kontermann, 2016; Pisal et al., 2010, Wang et al, 2007). Since PEG is non-degradable in circulation, it may lead to renal, hepatic and splenic vacuolization (Qi and Chilkoti, 2015; Pelegri-O'Day et al., 2014; Zhang et al., 2014). Immunological response subsequent to administration of PEGylated molecules is another prevailing issue (Swierczewska et al., 2015; Kontermann, 2016). Development of antibodies against PEGylated therapeutic products such as Krystexxa® (PEGylated Uricase) and

Oncospar® (PEGylated Asparginase) has been reported and may lead to accelerated clearance of the therapeutic (Armstrong, et al., 2007; Garay et al., 2012; Sundy et al., 2001; Swierczewska et al., 2015). In case of glycosylation, the performance of the therapeutic protein may be affected by aberrant glycosylation patterns that can further lead to rapid clearance through mannose- and asialoglycoprotien-receptors and in addition certain carbohydrate structures may lead to development of an immune response (Vugmeyester et al., 2012; Jenkins et al., 1994, 1996). In case of pharmaceutical formulation such as liposomes, their stability in blood is questionable due to low critical micelle concentration (CMC) (Landfester et al., 2012).

One of the important issues faced by protein therapeutics is the formulational stability of the finished product (Strohl, 2015). The critical aspect of formulation stability lies in poor solubility and tendency of chimeric proteins to form aggregates and micelles, however, the introduction of glycosylation sites in the chimera might help in overcoming aggregation (Huang and Swanson, 2013; Strohl, 2015). In addition, immunogenicity is the bottleneck for the application of therapeutic proteins (Baldo, 2015; De Groot and Scott, 2007; Jawa et al., 2013). It is crucial that the chimeric protein must elicit negligible to very low immune response, subsequent to administration, this is particularly important for chimeras developed for long-term therapy (Swierczewska et al., 2015; Strohl, 2015). Proteins are recognized by the immune system owing to the presence of T and B cell epitopes, in case of chimerized proteins, immunogenic response against the protein of interest could potentially worsen the disease condition in patients (Purcell and Lockey, 2008; Strohl, 2015). In addition, chimerization of proteins may lead to the formation of new epitopes that may elicit further immunogenic response (Strohl, 2015). Furthermore, several aspects relating to molecular structure and formulation affect the immunogenicity of therapeutic proteins (Purcell and Lockey, 2008; Schmidt, 2013a; Strohl, 2015; Strohl and Strohl, 2012). However, there have

been lesser incidences of immunogenic response and anti-drug antibody formation subsequent to administration of chimeric proteins (Strohl, 2015). One of the methods utilized to reduce immunogenicity, includes the prediction and elimination of T cell epitopes in a chimeric protein (Strohl, 2015). In addition, stability of finished product (such as optimal solubility and lack of aggregation) also help in eliminating the development of immunogenic response (Baldo, 2015; Jawa et al., 2013; Strohl, 2015; Strohl and Strohl 2012).

In case of Fc fusions, the issue of inconsistent glycosylation either in linker or in chimera and the functionality of Fc domain needs attention (Strohl, 2015). Orientation of the effector molecule may also play a key role, as the binding of effector towards *N*- or *C*-terminus of the HLE partner may significantly affect its activity (Schmidt, 2013a). Hence, an attempt for fusion of target proteins should be made at both *N*- and *C*-terminus, to design chimera with maximum activity. In addition, since linkers also affect the activity and utility of chimeric proteins, the choice of linkers (rigid, flexible and cleavable) should be made as per the desired therapeutic effect of the target protein (Chen et al, 2013b; Schmidt, 2013a). Moreover, research should also be focussed towards exploring the different avenues of linker design, to provide much wider variety and combinations.

Studies done on recombinant polymeric peptide repeats have been limited in contrast to Fc, HSA and transferrin fusion (Strohl, 2015). Therefore, much is unknown about these approaches and elaborate studies are required to establish them as candidates for HLEP, and since these platforms are unnatural repeats of amino acids, use of these approaches may also raise several multifaceted issues (Strohl, 2015). Approaches such as HAPylation and GLK fusion offer small half-life improvements, and mostly are in their incipient stages, and much study is required to establish a clinical basis for the use of these approaches (Strohl, 2015). In case of CTP fusion, owing to the strong negative charge, the biological activity of the chimera may be affected (Strohl, 2015). However, considering the half-life extension

afforded by the fusion with recombinant polymeric peptide repeats and CTP, their use seems to be more beneficial than approaches involving conjugation and encapsulation (Bar-Ilan et al., 2018; Gebauer and Skerra, 2018; Hershkovitz et al., 2016; Strasburger et al., 2017; Strohl, 2015).

Therefore, considering all the currently known facts, it seems that the protein chimerization, is more advantageous in comparison to other approaches for half-life extension (e.g., PEGylation, glycosylation, liposome formulation) for delivery of protein therapeutics.

Conclusion and Future Prospects

Biopharmaceuticals are clearly leading the way for pharmaceutical therapy. The number of approvals from Jan 2015 to July 2018 (~ 3.5 years) were almost double in comparison to approvals in each five-year span from 1995-2014 (Walsh, 2018). Interestingly, amongst the products approved between Jan 2014 to July 2018, >90% were protein therapeutics that include mAbs, clotting factors, enzymes and vaccines (Walsh, 2018). This suggests that protein therapeutics have become the cornerstone of biological therapy. Furthermore, since their inception, half-life extension technologies have come very far and with the development of newer approaches like Fc fusion, albumin fusion, Tf fusion etc., we now have wide variety of approaches to choose from (Strohl, 2015; Kontermann, 2012, 2016). However, even with such remarkable advancements there are considerable challenges that need to be addressed.

With the increasing discovery of novel pathophysiological mechanisms of various diseases, the significance of protein therapeutics in the current scenario, for disease interventions is more than ever before. However, the application of an emerging protein therapeutic may be hindered due to its poor pharmacokinetic attributes. This clearly

emphasizes that mere discovery may not be enough for most protein therapeutics, engineering them for optimum pharmacokinetic and pharmacodynamics is equally important. Furthermore, creation of computational methods, programs or software applications for the design and engineering of the chimera, with integrated systems for the prediction of immunogenicity, can ease out and accelerate the development of chimeric proteins with improved of pharmacokinetic and pharmacodynamic properties (Paladino et al., 2017; Wang et al., 2018). It is important to note that programs for designing linkers for chimeric proteins are already available, e.g., LINKER and SynLinker (Crasto and Feng, 2000; Liu et al., 2015). Such advancements in computational approaches may also lead to the development of 'tailored protein therapeutics' with customizable half-life, which may even have possible applications in personalized medicine. Finally, the development of multifunctional half-life extended chimeric proteins (with multiple effector proteins) for complex disease interventions must be explored in much detail (Chen et al., 2011; Jochems et al., 2019).

Acknowledgements

A.R.S.I. thanks The University of Newcastle for providing the resources to write the review. A.H.P. thanks the Department of Biotechnology, New Delhi, Government of India and NIPER, S.A.S. Nagar for providing support through a research grant (BT/PR23283/MED/30/1953/2018).

Authorship Contributions

Wrote or contributed to the writing of the manuscript: Iyengar, Gupta, Jawaleker and Pande

References

Abramson A, Caffarel-Salvador E, Khang M, Dellal D, Silverstein D, Gao Y, Frederiksen MR, Vegge A, Hubálek F, Water JJ, Friderichsen AV, Fels J, Kirk RK, Cleveland C, Collins J, Tamang S, Hayward A, Landh T, Buckley ST, Roxhed N, Rahbek U, Langer R and Traverso G (2019) An ingestible self-orienting system for oral delivery of macromolecules. *Science* **363**: 611-615.

Aghaabdollahian S, Cohan RA, Norouzian D, Davami F, Karam MRA, Torkashvand F, Vaseghi G, Moazzami R, Dizaji SL (2019) Enhancing bioactivity, physicochemical, and pharmacokinetic properties of a nano-sized, anti-VEGFR2 Adnectin, through PASylation technology. *Sci Rep.* **9**: 2978, 1-12.

AlQahtani AD, Al-Mansoori L, Bashraheel SS, Rashidi FB, Al-Yafei A, Elsinga P, Domling A, and Goda SK. (2019) Production of "biobetter" glucarpidase variants to improve drug detoxification and antibody directed enzyme prodrug therapy for cancer treatment. *Eur J Pharm Sci* **127**: 79-91.

Alters SE, McLaughlin B, Spink B, Lachinyan T, Wang CW, Podust V, Schellenberger V, Stemmer WP (2012) GLP2-2G-XTEN: a pharmaceutical protein with improved serum half-life and efficacy in a rat Crohn's disease model. *PLoS One* **7** :e50630.

Amet N, Lee HF and, Shen WC (2009) Insertion of the designed helical linker led to increased expression of tf-based fusion proteins. *Pharm Res* **26**: 523-528.

Andersen JT, Dalhus B, Viuff D, Ravn BT, Gunnarsen KS, Plumridge A, Bunting K, Antunes F, Williamson R, Athwal S, Allan E, Evans L, Bjørås M, Kjærulff S, Sleep D, Sandlie I, and Cameron J (2014) Extending serum half-life of albumin by engineering neonatal Fc receptor (FcRn) binding. *J Biol Chem* **289**: 13492-13502.

Armstrong JK, Hempel G, Koling S, Chan LS, Fisher T, Meiselman HJ, and Garratty G (2007) Antibody against poly(ethylene glycol) adversely affects PEG-asparaginase therapy in acute lymphoblastic leukemia patients. *Cancer* **10**: 103-111.

Argos P (1990) An investigation of oligopeptides linking domains in protein tertiary structures and possible candidates for general gene fusion. *J Mol Biol* **211**: 943-958.

Bai Y, Ann DK, and Shen WC (2005) Recombinant granulocyte colony-stimulating factor-transferrin fusion protein as an oral myelopoietic agent. *Proc. Natl. Acad. Sci. U. S. A.* **102**: 7292–7296.

Bai Y, and Shen WC (2006). Improving the oral efficacy of recombinant granulocyte colony-stimulating factor and transferrin fusion protein by spacer optimization. *Pharm Res,* **23**: 2116-2121.

Baker M, Reynolds HM, Lumicisi B, and Bryson, CJ (2010). Immunogenicity of protein therapeutics: the key causes, consequences and challenges. *Self/Nonself* **1**: 314-322.

Baldo BA (2015) Chimeric fusion proteins used for therapy: indications, mechanisms, and safety. *Drug Saf* **38**: 455-479.

Bar-Ilan A, Livnat T, Hoffmann M, Binder L, Zakar M, Guy R, Felikman Y, Moschcovich L, Shenkman B, Monroe D, Hershkovitz O, Kenet G, and Hart G (2018) In vitro characterization of MOD-5014, a novel long-acting carboxy-terminal peptide (CTP)-modified activated FVII. *Haemophilia* **4**: 477-486.

Bas M, Terrier A, Jacque E, Dehenne A, Pochet-Béghin V, Beghin C, Dezetter AS, Dupont G, Engrand A, Beaufils B, Mondon P, Fournier N, de Romeuf, Jorieux S, Fontayne A, Mars LT, and Monnet C (2019) Fc sialylation prolongs serum half-life of therapeutic antibodies. *J Immunol* **202**: 1582-1594.

Binder U, and Skerra A (2017) PASylation®: A versatile technology to extend drug delivery. *Curr Opin Colloid Interface Sci* **31**: 10-17.

Bird RE, Hardman KD, Jacobson JW, Johnson S, Kaufman BM, Lee SM, Lee T, Pope SH, Riordan GS, and Whitlow M. (1988) Single-chain antigen-binding proteins. *Science* **242**: 423-426.

Blackmore E (2017) Human-pig hybrid created in the lab-here are the facts, National Geographic (https://news.nationalgeographic.com/2017/01/human-pig-hybrid-embryo-chimera-organs-health-science/) (Retrieved 31-01-2019).

Boado RJ, Zhang Y, Zhang Y, Wang Y, and Pardridge WM (2008). IgG-Paraoxonase-1 Fusion Protein for Targeted Drug Delivery across the Human Blood-Brain Barrier. *Mol Pharm* **5**: 1037-1043.

Bond A (2006). Exenatide (Byetta) as a novel treatment option for type 2 diabetes mellitus. *Proc (Bayl Univ Med Cent)* **19**: 281-284.

Breibeck J, and Skerra A (2017) The polypeptide biophysics of proline/alanine-rich sequences (PAS): Recombinant biopolymers with PEG-like properties. *Biopolymers*. **109**: 1-12.

Bush MA, Matthews JE, De Boever EH, Dobbins RL, Hodge RJ, Walker SE, Holland MC, Gutierrez M, and Stewart MW (2009) Safety, tolerability, pharmacodynamics and pharmacokinetics of albiglutide, a longacting glucagon-like peptide-1 mimetic, in healthy subjects. *Diabetes Obes Metab* **11**: 498–505.

Calo D, Hart G, Hoffman M, Yagev LI, Tzur Y, Binder L, Monahan P, Zakar M, Guy R, Felikman Y, Moschcovich L, Bar-Ilan A, and Hershkovitz O (2015) Enhancing the longevity and in vivo potency of therapeutic proteins: the power of CTP. *Precision Medicine* **2**: 1-8.

Castonguay R, Lachey J, Wallner S, Strand J, Liharska K, Watanabe AE, Cannell M, Davies MV, Sako D, Troy ME, Krishnan L, Mulivor AW, Li H, Keates S, Alexander MJ,

Pearsall RS, and Kumar R (2019) Follistatin-288-Fc Fusion Protein Promotes Localized Growth of Skeletal Muscle. *J Pharmacol Exp Ther* **368**: 435-445.

Chen X, Bai Y, Zaro J, and Shen WC (2010) Design of an *in vivo* cleavable disulfide linker in recombinant fusion proteins. *Biotechniques* **49**: 513-518.

Chen X, Deng J, Cui W, Hou S, Zhang J, Zheng X, Ding X, Wei H, Zhou Z, Kim K, Zhan CG, and Zheng F (2018) Development of Fc-fused Cocaine Hydrolase for cocaine addiction treatment: catalytic and pharmacokinetic properties. *AAPS J* **20**: 53.

Chen X, Lee H-F, Zaro JL, and Shen WC. (2011) Effects of receptor binding on plasma half-life of bifunctional transferrin fusion proteins. *Mol Pharm* **8**: 457-465.

Chen X, Zaro J, and Shen W-C (2013a) Fusion Protein Linkers: Effects on Production, Bioactivity, and Pharmacokinetics, in *Fusion protein technologies for biopharmaceuticals: applications and challenges* (Schmidt SR ed) pp 57-74, John Wiley & Sons, Inc., New Jersey, USA.

Chen X, Zaro JL, and Shen WC (2013b) Fusion protein linkers: property, design and functionality. *Adv Drug Del Rev* **65**: 1357-1369.

Chia J, Louber J, Glauser I, Taylor S, Bass GT, Dower SK, Gleeson PA and, Verhagen AM (2018) Half-life-extended recombinant coagulation factor IX-albumin fusion protein is recycled via the FcRn-mediated pathway. *J Biol Chem* **293**: 6363-6373.

Chirmule N, Jawa V, and Meibohm B (2012) Immunogenicity to therapeutic proteins: impact on PK/PD and efficacy. *AAPS J* **14**: 296-302.

Cleland JL, Geething NC, Moore JA, Rogers BC, Spink BJ, Wang CW, Alters SE, Stemmer WP, and Schellenberger V. (2012) A novel long-acting human growth hormone fusion protein, (VRS-317): enhanced in vivo potency and half-life. *J Pharm Sci* **101**: 2744-2754.

Colletier JP, Chaize B, Winterhalter M, and Fournier D (2002) Protein encapsulation in liposomes: efficiency depends on interactions between protein and phospholipid bilayer. *BMC Biotechnol* **2**: 9.

Conrad U, Plagmann I, Malchow S, Sack M, Floss DM, Kruglov AA, Nedospasov SA, Rose-John S, and Scheller J. (2011) ELPylated anti-human TNF therapeutic single-domain antibodies for prevention of lethal septic shock. *Plant Biotechnol* **9**: 22-31.

Crasto CJ, and Feng JA (2000) LINKER: a program to generate linker sequences for fusion proteins. *Protein Eng Des Sel.* **13**: 309-312.

Czajkowsky DM, Hu J, Shao Z, and Pleass RJ (2012) Fc-fusion proteins: new developments and future prospects. *EMBO Mol Med* **4**:1015-1028.

Datta-Mannan A, Boyles J, Huang L, Jin ZY, Peariso A, Murphy AT, Ellis B, Douglass N, Norouziyan-Cooper F, Witcher DR (2019) Engineered FcRn binding fusion peptides significantly enhance the half-life of a Fab domain in cynomolgus monkeys. *Biotechnol J* **14**: e1800007, 1-12.

De Kruijf W and Ehrhardt C (2017) Inhalation delivery of complex drugs - the next steps. *Curr Opin Pharmacol* **36**: 52-57.

De Groot AS, and Scott DW (2007) Immunogenicity of protein therapeutics. *Trends Immunol* **28**: 482-490.

De Meyts P (2017) Early Recombinant Protein Therapeutics, in *Protein Therapeutics*, Vol. 1 (Vaughan T, Osbourn J, and Jallal B eds) pp 1-19, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany.

Deen WM, Lazzara MJ, and Myers BD (2001) Structural determinants of glomerular permeability *Am J. Physiol Renal Physiol* **281**: F579 – F596.

Diao L, and Meibohm B (2013) Pharmacokinetics and pharmacokinetic-pharmacodynamic correlations of therapeutic peptides. *Clin Pharmacokinet* **52**: 855-868.

Dirks NL, and Meibohm B (2010) Population pharmacokinetics of therapeutic monoclonal antibodies. *Clin Pharmacokinet* **49**: 633-659.

Dunsford I, Bowley CC, Hutchison AM, Thompson JS, and Sanger R (1953) A human blood-group chimera. *Br Med J* **2**: 81.

Eckardt S, McLaughlin KJ, and Willenbring H (2011) Mouse chimeras as a system to investigate development, cell and tissue function, disease mechanisms and organ regeneration. *Cell Cycle* **10**: 2091-2099.

Fallon J, Tighe R, Kradjian G, Guzman W, Bernhardt A, Neuteboom B, and Greiner JW (2014) The immunocytokine NHS-IL12 as a potential cancer therapeutic. *Oncotarget* **5**: 1869-1884.

Fares F (2012) Half-Life extension through O-Glycosylation, in *Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives* (Kontermann RE ed) pp 81-94, Wiley-VCH Verlag & Co.,Weinheim, Germany.

Fares F, and Azzam N (2019) Development of long-acting recombinant glycoprotein hormones by increasing the carbohydrate content. *Drug Discov Today* doi: 10.1016/j.drudis.2019.01.017.

Fares F, Guy R, Bar-Ilan A, Felikman Y, and Fima E (2010) Designing a long-acting human growth hormone (hGH) by fusing the carboxyl-terminal peptide of human chorionic gonadotropin beta-subunit to the coding sequence of hGH. *Endocrinology* **151**: 4410-4417.

Fares FA, Suganuma N, Nishimori K, LaPolt PS, Hsueh AJ, and Boime I (1992) Design of a longacting follitropin agonist by fusing the C-terminal sequence of the chorionic gonadotropin beta subunit to the follitropin beta subunit. *Proc Natl Acad Sci USA* **89**: 4304-4308.

Ferrarese M, Pignani S, Lombardi S, Balestra D, Bernardi F, Pinotti M, and Branchini A (2019) The carboxyl-terminal region of human coagulation factor X as a natural linker for fusion strategies. *Thromb Res* **173**: 4-11.

Floss DM, Conrad U, Rose-John S, and Scheller J (2013) ELP-fusion technology for biopharmaceuticals, in *Fusion protein technologies for biopharmaceuticals: applications and challenges* (Schmidt SR ed) pp 211-216, John Wiley & Sons, Inc., New Jersey, USA.

Floss DM, Schallau K, Rose-John S, Conrad U and Scheller J. (2010) Elastin-like polypeptides revolutionize recombinant protein expression and their biomedical application. *Trends Biotechnol* **28**: 37-45.

Fontaine-Pérus J (1999) 7 Mouse-Chick Chimera: An experimental system for study of somite development. *Curr Top Dev Biol* **48**: 269-300.

Fuchs H and Igney F (2017) Binding to Ocular Albumin as a Half-Life Extension Principle for Intravitreally Injected Drugs: Evidence from Mechanistic Rat and Rabbit Studies. *J Ocul Pharmacol Ther* **33**: 115-122.

Garay RP, El-Gewely R, Armstrong JK, Garratty G, and Richette P (2012) Antibodies against polyethylene glycol in healthy subjects and in patients treated with PEG-conjugated agents. *Expert Opin Drug Deliv* **9**: 1319-1323.

Gebauer M and Skerra A (2018) Prospects of PASylation® for the design of protein and peptide therapeutics with extended half-life and enhanced action. *Bioorg Med Chem* **26**: 2882-2887.

Gellissen G (2005) Production of Recombinant Proteins: Novel Microbial and Eukaryotic Expression Systems, Wiley-VCH Verlag GmbH & Co., Weinheim, Germany.

Glaesner W, Vick AM, Millican R, Ellis B, Tschang SH, Tian Y, Bokvist K, Brenner M, Koester A, Porksen N, Etgen G, and Bumol T (2010) Engineering and characterization

of the long-acting glucagon-like peptide-1 analogue LY2189265, an Fc fusion protein. *Diabetes Metab Res Rev* **26**:287-296.

Gokhale R.S. and Khosla C. (2000). Role of linkers in communication between protein modules. *Curr Opin Chem Biol* **4**: 22-27.

Goodall LJ, Ovecka M, Rycroft D, Friel SL, Sanderson A, Mistry P, Davies ML, and Stoop AA (2015) Pharmacokinetic and pharmacodynamics characterisation of an anti-mouse TNF receptor 1 domain antibody formatted for in vivo half-life extension. *PLoS One* **10**: e137065, 1-16.

Graf L (2018) Extended Half-Life Factor VIII and Factor IX Preparations. *Transfus Med Hemother* **45**: 86-91.

Harari D, Kuhn N, Abramovich R, Sasson K, Zozulya AL, Smith P, Schlapschy M, Aharoni R, Köster M, Eilam R, Skerra A, and Schreiber G (2014) Enhanced in vivo efficacy of a type I interferon superagonist with extended plasma half-life in a mouse model of multiple sclerosis. *J Biol Chem* **289**: 29014-29029.

Hartung A, and Bendas G (2012) Half-Life Extension with Pharmaceutical Formulations: Liposomes, in *Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives* (Kontermann RE ed) pp 299-314, Wiley-VCH Verlag & Co.,Weinheim, Germany.

Hassouneh W, MacEwan SR, and Chilkoti A (2012) Fusions of elastin-like polypeptides to pharmaceutical proteins. *Methods Enzymol* **502**: 215-237.

Hershkovitz O, Bar-Ilan A, Guy R, Felikman Y, Moschcovich L, Hwa V, Rosenfeld RG, Fima E, and Hart G. (2016*) In Vitro* and *in vivo* characterization of MOD-4023, a long-acting carboxy-terminal peptide (CTP)-modified human growth hormone. *Mol Pharm* **13**: 631-639.

Hoogenboezem EN, and Duvall CL (2018) Harnessing albumin as a carrier for cancer therapies. *Adv Drug Deliv Rev* **130**: 73-89.

Hou Y, Zhou Y, Wang H, Sun J, Wang R, Sheng K, Yuan J, Hu Y, Chao Y, Liu Z, and Lu H (2019) Therapeutic protein PEPylation: The helix of nonfouling synthetic polypeptides minimizes antidrug antibody generation. *ACS Cent Sci*. **5**: 229-236.

Huang C (2009) Receptor-Fc fusion therapeutics, traps, and MIMETIBODY technology. *Curr Opin Biotechnol* **20**: 692-699.

Huang C, and Swanson VR (2013) Peptide-Fc fusion therapeutics: applications and challenges, in *Fusion protein technologies for biopharmaceuticals: applications and challenges* (Schmidt SR ed) pp 123-142, John Wiley & Sons, Inc., New Jersey, USA.

Huang YS, Wen XF, Wu YL, Wang YF, Fan M, Yang ZY, Liu W, and Zhou LF (2010) Engineering a pharmacologically superior form of granulocyte-colony-stimulating factor by fusion with gelatin-like protein polymer. *Eur J Pharm Biopharm* **72**: 435-441.

Ikeda T, Tennyson RL, Walker SN, Harris RS, and McNaughton BR (2019) Evolved proteins inhibit entry of Enfuvirtide-resistant HIV-1. *ACS Infect Dis.* doi: 10.1021/acsinfecdis.8b00362.

Jacobs SA, Gibbs AC, Conk M, Yi F, Maguire D, Kane C, and O'Neil KT (2015) Fusion to a highly stable consensus albumin binding domain allows for tunable pharmacokinetics. *Protein Eng Des Sel* **28**:385-393.

Jank L, Pinto-Espinoza C, Duan Y, Koch-Nolte F, Magnus T, and Rissiek B (2019) Current approaches and future perspectives for nanobodies in stroke diagnostic and therapy. *Antibodies* **8**: 1-17.

Jawa V, Cousens L and De Groote AS (2013) Immunogenicity of therapeutic fusion proteins: contributory factors and clinical experience, in *Fusion protein technologies for biopharmaceuticals: applications and challenges* (Schmidt SR ed) pp 150–175, John Wiley & Sons, Inc., New Jersey, USA.

Jazayeri JA, and Carroll GJ (2008) Fc-based cytokines: prospects for engineering superior therapeutics. *BioDrugs* **22**: 11-26.

Jenkins N, and Curling EM (1994) Glycosylation of recombinant proteins: problems and prospects. *Enzyme Microb Technol* **16**: 354-364.

Jenkins N, Parekh RB, and James DC (1996) Getting the glycosylation right: implications for the biotechnology industry. *Nat Biotechnol* **14**: 975-981.

Jevševar S, and Kunstelj M (2012) Half-life extension through PEGylation, in *Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives* (Kontermann RE ed) pp 41-62, Wiley-VCH Verlag & Co.,Weinheim, Germany.

Jochems C, Tritsch SR, Knudson KM, Gameiro SR, Rumfield CS, Pellom ST, Morillon YM, Newman R, Marcus W, Szeto C, Rabizadeh S, Wong HC, Soon-Shiong P, Schlom J (2019) The multi-functionality of N-809, a novel fusion protein encompassing anti-PD-L1 and the IL-15 superagonist fusion complex. *Oncoimmunology* **8**: e1532764, 1-15.

Keizer RJ, Huitema AD, Schellens JH, and Beijnen JH (2010) Clinical pharmacokinetics of therapeutic monoclonal antibodies. *Clin Pharmacokinet* **49**: 493-507.

Khodabakhsh F, Behdani M, Rami A and Kazemi-Lomedasht F (2018) Single-domain antibodies or nanobodies: a class of next-generation antibodies. *Int Rev Immunol* **37**: 316-322.

Kim BJ, Zhou J, Martin B, Carlson OD, Maudsley S, Greig NH, Mattson MP, Ladenheim EE, Wustner J, Turner A, Sadeghi H, and Egan JM. (2010) Transferrin fusion technology: a novel approach to prolonging biological half-life of insulinotropic peptides. *J Pharmacol Exp Ther* **334**: 682-692.

Kim J, Hayton WL, Robinson JM, and Anderson CL (2007) Kinetics of FcRn-mediated recycling of IgG and albumin in human: pathophysiology and therapeutic implications using a simplified mechanism-based model. *Clin Immunol* **122**: 146-155.

Kimchi-Sarfaty C, Alexaki A, and Sauna ZE (2017) Introduction, in Protein Therapeutics (Sauna ZE, and Kimchi-Sarfaty C eds), pp v-xii, Springer International Publishing AG, Cham, Switzerland.

Kontermann RE (2011) Strategies for extended serum half-life of protein therapeutics. *Curr Opin Biotechnol* **22**: 868-876.

Kontermann RE (2012) Half-life modulating strategies - an Introduction, in *Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives* (Kontermann RE ed) pp 3-23, Wiley-VCH Verlag & Co.,Weinheim, Germany.

Kontermann RE (2016) Half-life extended biotherapeutics. *Expert Opin Biol Ther* **16**: 903-915.

Krishnamurthy S, Muthukumaran P, Jayakumar MKG, Lisse D, Masurkar ND,Xu C, Chan JM, Drum CL (2019) Surface protein engineering increases circulation time of cell membrane-based Nanotherapeutic. *Nanomedicine* doi: 10.1016/j.nano.2019.02.024.

Kuo TT, and Aveson VG (2011) Neonatal Fc receptor and IgG-based therapeutics. *MAbs* **3**: 422-430.

Lagassé, HD, Alexaki A, Simhadri VL, Katagiri NH, Jankowski W, Sauna ZE, and Kimchi-Sarfaty C (2017) Recent advances in (therapeutic protein) drug development. *F1000Res* **6**: 113.

Landfester K, Musyanovych A, and Mailänder V (2012) Half-Life extension with pharmaceutical formulations: nanoparticles by the miniemulsion process, in *Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives* (Kontermann RE ed) pp 315-340, Wiley-VCH Verlag & Co.,Weinheim, Germany.

Larsen MT, Kuhlmann M, Hvam ML, and Howard KA (2016) Albumin-based drug delivery: harnessing nature to cure disease. *Mol Cell Ther* **4**: 3.

Larsen MT, Rawsthorne H, Schelde KK, Dagnæs-Hansen F, Cameron J, and Howard KA (2018) Cellular recycling-driven in vivo half-life extension using recombinant albumin fusions tuned for neonatal Fc receptor (FcRn) engagement. *J Control Release* **287**: 132-141.

Leader B, Baca QJ, and Golan DE (2008) Protein therapeutics: a summary and pharmacological classification. *Nat Rev Drug Discov* **7**: 21-39.

Lee ES, and YS Youn (2016) Albumin-based potential drugs: focus on half-life extension and nanoparticle preparation. *J Pharm Investig* **46**: 305-315.

Li B, Chen A, Zou S, Wu J, Wang H, Chen R, and Luo M (2019) Albumin fusion improves the pharmacokinetics and in vivo antitumor efficacy of canine interferon gamma. *Int J Pharm* **58**: 404-412.

Li H, and d'Anjou M (2009) Pharmacological significance of glycosylation in therapeutic proteins. *Curr Opin Biotechnol* **20**: 678-684.

Li H, and Qian ZM (2002) Transferrin/transferrin receptor-mediated drug delivery. *Med Res Rev* **22**: 225-230.

Li R, Yang H, Jia D, Nie Q, Cai H, Fan Q, Wan L, Li L, and Lu X (2016) Fusion to an albumin-binding domain with a high affinity for albumin extends the circulatory half-life and enhances the in vivo antitumor effects of human TRAIL. *J Control Release* **228**: 96-106.

Liu C, Chin JX, and Lee DY (2015) SynLinker: an integrated system for designing linkers and synthetic fusion proteins. *Bioinformatics* **31**: 3700-3702.

Liu L (2018) Pharmacokinetics of monoclonal antibodies and Fc-fusion proteins. *Protein Cell* **9**: 15-32.

MacEwan SR, and Chilkoti A (2014) Applications of elastin-like polypeptides in drug delivery. *J Control Release* **190**: 314-330.

Mager DE (2006) Target-mediated drug disposition and dynamics. *Biochem. Pharmacol*. **72**: 1-10.

Mahlangu J, Young G, Hermans C, Blanchette V, Berntorp E, and Santagostino E (2018) Defining extended half-life rFVIII-A critical review of the evidence. *Haemophilia* **24**: 348-358.

McDonagh CF, Huhalov A, Harms BD, Adams S, Paragas V, Oyama S, Zhang B, Luus L, Overland R, Nguyen S, Gu J, Kohli N, Wallace M, Feldhaus MJ, Kudla AJ, Schoeberl B, and Nielsen UB (2012) Antitumor activity of novel bispecific antibody that targets the ErbB2/ErbB3 oncogenic unit and inhibits heregulin-induced activation of ErbB3. *Mol Cancer Ther* **11**: 582-593.

Meibohm B (2012) Pharmacokinetics and half-life of protein therapeutics, in *Therapeutic Proteins: Strategies to Modulate Their Plasma Half-Lives* (Kontermann RE ed) pp 23-38, Wiley-VCH Verlag & Co.,Weinheim, Germany.

Meibohm B and Braeckman RA (2007) Pharmacokinetics and pharmacodynamics of peptides and proteins, in *Pharmaceutical Biotechnology: Concepts and Applications* (Crommelin DJA, Sindelar RD, and Meibohm B eds), pp 95-123, Informa Healthcare, New York, USA.

Moore WV, Nguyen HJ, Kletter GB, Miller BS, Rogers D, Ng D, Moore JA, Humphriss E, Cleland JL, and Bright GM (2016) A randomized safety and efficacy study of somavaratan (VRS-317), a long-acting rhGH, in pediatric growth hormone deficiency. *J Clin Endocrinol Metab* **101**: 1091-1097.

Müller D, Karle A, Meissburger B, Höfig I, Stork R, and Kontermann RE (2007) Improved pharmacokinetics of recombinant bispecific antibody molecules by fusion to human serum albumin. *J Biol Chem* **282**: 12650-12660.

Norris R, Smith RH, and Vaughn KC (1983) Plant chimeras used to establish de novo origin of shoots. *Science* **220**: 75-76.

Paladino A, Marchetti F, Rinaldi S, and Colombo G (2017) Protein design: from computer models to artificial intelligence. *WIREs Comput Mol Sci* **7**: e1318, 1-21.

Pardridge WM (2015) Blood-brain barrier drug delivery of IgG fusion proteins with a transferrin receptor monoclonal antibody. *Expert Opin Drug Deliv* **12**: 207-222.

Peck HT (1898) Chimaera, in *Harpers Dictionary of Classical Antiquities* (Peck HT ed), Harper and Brothers, New York, USA (http://www.perseus.tufts.edu/hopper/text?doc=Perseus:text:1999.04.0062:entry=chimaera-harpers) (Retrieved 31-01-2019).

Pelegri-O'Day EM, Lin EW, and Maynard HD (2014) Therapeutic protein-polymer conjugates: advancing beyond PEGylation. *J Am Chem Soc* **136**: 14323-14332.

Pisal DS, Kosloski MP, and Balu-Iyer SV (2010) Delivery of therapeutic proteins. *J Pharm Sci* **99**: 2557-2575.

Podust VN, Sim BC, Kothari D, Henthorn L, Gu C, Wang CW, McLaughlin B, and Schellenberger V (2013) Extension of *in vivo* half-life of biologically active peptides via chemical conjugation to XTEN protein polymer. *Protein Eng Des Sel* **26**: 743-753.

Podust VN, Balan S, Sim BC, Coyle MP, Ernst U, Peters RT, and Schellenberger V (2016) Extension of in vivo half-life of biologically active molecules by XTEN protein polymers. *J Control Release* **240**: 52-66.

Presta LG (2008) Molecular engineering and design of therapeutic antibodies. *Curr Opin Immunol* **20**: 460-470.

Purcell RT, and Lockey RF (2008) Immunologic responses to therapeutic biologic agents. *J Investig Allergol Clin Immunol* **18**: 335-342.

Qi Y, and Chilkoti A (2015). Protein-polymer conjugation-moving beyond PEGylation. *Curr Opin in Chem Biol* **28**: 181-193.

Ramírez-Andersen HS, Behrens C, Buchardt J, Fels JJ, Folkesson CG, Jianhe C, Nørskov-Lauritsen L, Nielsen PF, Reslow M, Rischel C, Su J, Thygesen P, Wiberg C, Zhao X, Wenjuan X, and Johansen NL (2018) Long-Acting Human Growth Hormone Analogue by Noncovalent Albumin Binding. *Bioconjug Chem* **29**: 3129-3143.

Rath T, Baker K, Dumont JA, Peters RT, Jiang H, Qiao SW, Lencer WI, Pierce GF, and Blumberg RS (2015) Fc-fusion proteins and FcRn: structural insights for longer-lasting and more effective therapeutics. *Crit Rev Biotechnol* **35**: 235-254.

Rattanapisit K, Srifa S, Kaewpungsup P, Pavasant P, and Phoolcharoen W (2019) Plant-produced recombinant Osteopontin-Fc fusion protein enhanced osteogenesis. *Biotechnol Rep* **20**: e00312, 1-6.

Richter F, Zettlitz KA, Seifert O, Herrmann A, Scheurich P, Pfizenmaier K, and Kontermann RE (2019) Monovalent TNF receptor 1-selective antibody with improved affinity and neutralizing activity. *MAbs* **11**: 166-177.

Robbie GJ, Criste R, Dall'acqua WF, Jensen K, Patel NK, Losonsky GA, and Griffin MP (2013). A novel investigational Fc modified humanized monoclonal antibody, motavizumab-YTE, has an extended half-life in healthy adults. *Antimicrob Agents Chemother* **57**: 6147-6153.

Rogers B, Dong D, Li Z, and Li Z (2015) Recombinant human serum albumin fusion proteins and novel applications in drug delivery and therapy. *Curr Pharm Des* **21**:1899-1907.

Roopenian DC, and Akilesh S. (2007) FcRn: the neonatal Fc receptor comes of age. *Nat Rev Immunol* **7**: 715-725.

Rycroft D, and Holt LJ (2012) Methods for determining the PK parameters of AlbudAbs^{TM} and of long serum half-life drugs made using the AlbudAb^{TM} technology. *Methods Mol Biol* **911**:457-473.

Sabourin M, Tuzon C, Fisher T, and Zakian V (2007) A flexible protein linker improves the function of epitope-tagged proteins in Saccharomyces cerevisiae. *Yeast* **24**: 39-45.

Sand KM, Bern M, Nilsen J, Dalhus B, Gunnarsen KS, Cameron J, Grevys A, Bunting K, Sandlie I, and Andersen JT (2014) Interaction with both domain I and III of albumin is required for optimal pH-dependent binding to the neonatal Fc receptor (FcRn). *J Biol Chem* **289**: 34583-34594.

Sand KM, Bern M, Nilsen J, Noordzij HT, Sandlie I, and Andersen JT (2015) Unraveling the interaction between FcRn and albumin: opportunities for design of albumin-based therapeutics. *Front Immunol* **5**: 682.

Santagostino E, Martinowitz U, Lissitchkov T, Pan-Petesch B, Hanabusa H, Oldenburg J, Boggio L, Negrier C, Pabinger I, von Depka Prondzinski M, Altisent C, Castaman G, Yamamoto K, Álvarez-Roman MT, Voigt C, Blackman N, Jacobs I, and PROLONG-9FP Investigators Study Group (2016) Long-acting recombinant coagulation factor IX albumin fusion protein (rIX-FP) in hemophilia B: results of a phase 3 trial. *Blood* **127**: 1761-1769.

Santelices B (2004) Mosaicism and chimerism as components of intraorganismal genetic heterogeneity. *J Evol Biol* **17**: 1187-1188.

Schellenberger V, Wang CW, Geething NC, Spink BJ, Campbell A, To W, Scholle MD, Yin Y, Yao Y, Bogin O, Cleland JL, Silverman J, and Stemmer WP. (2009) A recombinant polypeptide extends the in vivo half-life of peptides and proteins in a tunable manner. *Nat Biotechnol* **27**: 1186-1190.

Schlapschy M, Theobald I, Mack H, Schottelius M, Wester HJ, and Skerra A (2007) Fusion of a recombinant antibody fragment with a homo-amino-acid polymer: effects on biophysical properties and prolonged plasma half-life. *Protein Eng Des Sel* **20**: 273-284.

Schlapschy M, Binder U, Börger C, Theobald I, Wachinger K, Kisling S, Haller D, and Skerra A (2013) PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins. *Protein Eng Des Sel* **26**: 489-501.

Schmidt EM, Davies M, Mistry P, Green P, Giddins G, Feldmann M, Stoop AA, and Brennan FM (2013) Selective blockade of tumor necrosis factor receptor I inhibits proinflammatory cytokine and chemokine production in human rheumatoid arthritis synovial membrane cell cultures. *Arthritis Rheumat* **65**: 2262–2273.

Schmidt SR (2013a) Fusion protein technologies for biopharmaceuticals: applications and challenges, in *Fusion protein technologies for biopharmaceuticals: applications and challenges* (Schmidt SR ed) pp 3-24, John Wiley & Sons, Inc., New Jersey, USA.

Schmidt SR. (2013b) Fusion protein technologies for half-life extension, in *Fusion protein technologies for biopharmaceuticals: applications and challenges* (Schmidt SR ed) pp 93-106, John Wiley & Sons, Inc., New Jersey, USA.

Schoch A, Kettenberger H, Mundigl O, Winter G, Engert J, Heinrich J, and Emrich T (2015) Charge-mediated influence of the antibody variable domain on FcRn-dependent pharmacokinetics. *Proc Natl Acad Sci USA* **112**: 5997-6002.

Schulte S. (2009) Half-life extension through albumin fusion technologies. *Thromb Res* **124 Suppl. 2**: S6–S8.

Seijsing J, Sobieraj AM, Keller N, Shen Y, Zinkernagel AS, Loessner MJ, and Schmelcher M (2018) Improved biodistribution and extended serum half-life of a bacteriophage endolysin by albumin binding domain fusion. *Front Microbiol* **9**: 2927.

Shao J, Zaro JL, and Shen WC (2016) Proinsulin-Transferrin fusion protein exhibits a prolonged and selective effect on the control of hepatic glucose production in an experimental model of type 1 diabetes. *Mol Pharm* **13**: 2641-2646.

Shapiro AD, Pasi KJ, Ozelo MC, Kulkarni R, Barnowski C, Winding B, Szamosi J, and Lethagen S (2019) Extending recombinant factor IX Fc fusion protein dosing interval to 14 or more days in patients with hemophilia B. *Res Pract Thromb Haemost* **3**: 109-113.

Shapiro AD, Ragni MV, Valentino LA, Key NS, Josephson NC, Powell JS, Cheng G, Thompson AR, Goyal J, Tubridy KL, Peters RT, Dumont JA, Euwart D, Li L, Hallén B, Gozzi P, Bitonti AJ, Jiang H, Luk A, and Pierce GF (2012) Recombinant factor IX-Fc fusion protein (rFIXFc) demonstrates safety and prolonged activity in a phase 1/2a study in hemophilia B patients. *Blood* **119**: 666-672.

Sheffield WP, Eltringham-Smith LJ, and Bhakta V (2018) Fusion to Human Serum Albumin Extends the Circulatory Half-Life and Duration of Antithrombotic Action of the Kunitz Protease Inhibitor Domain of Protease Nexin 2. *Cell Physiol Biochem* **45**: 772-782.

Sinclair AM, and Elliott S (2005) Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. *J Pharm Sci* **94**: 1626-1635.

Sleep D (2015) Albumin and its application in drug delivery. *Expert Opin Drug Deliv* **12**: 793-812.

Sleep D, Cameron J, and Evans LR (2013) Albumin as a versatile platform for drug half-life extension. *Biochim Biophys Acta* **1830**: 5526-5534.

Sockolosky JT, and Szoka FC (2015) The neonatal Fc receptor, FcRn, as a target for drug delivery and therapy. *Adv Drug Deliv Rev* **91**: 109-124.

Solá RJ, and Griebenow K (2010) Glycosylation of therapeutic proteins: an effective strategy to optimize efficacy. *BioDrugs* **24**: 9-21.

Souders CA, Nelson SC, Wang Y, Crowley AR, Klempner MS and, Thomas W Jr (2015) A novel in vitro assay to predict neonatal Fc receptor-mediated human IgG half-life. *MAbs* **7**: 912-921.

Steiner D, Merz FW, Sonderegger I, Gulotti-Georgieva M, Villemagne D, Phillips DJ, Forrer P, Stumpp MT, Zitt C, and Binz HK (2017) Half-life extension using serum albumin-binding DARPin® domains. *Protein Eng Des Sel* **30**: 583-591.

Strasburger CJ, Vanuga P, Payer J, Pfeifer M, Popovic V, Bajnok L, Góth M, Olšovská V, Trejbalová L, Vadasz J, Fima E, Koren R, Amitzi L, Bidlingmaier M, Hershkovitz O, Hart G, and Biller BM (2017) MOD-4023, a long-acting carboxy-terminal peptide-modified human growth hormone: results of a Phase 2 study in growth hormone-deficient adults. *Eur J Endocrinol* **176**: 283-294.

Strohl WR (2015). Fusion proteins for half-life extension of biologics as a strategy to make biobetters. *BioDrugs* **29**: 215-239.

Strohl WR, and Strohl LM (2012) Development issues: antibody stability, developability, immunogenicity, and comparability, in *Therapeutic antibody engineering: current and future advances driving the strongest growth area in the pharma industry* (Strohl WR, and Strohl LM eds), pp 377-403, Woodhead Publishing Series in Biomedicine No. 11, Woodhead Publishing, Cambridge, USA.

Sun J and Michaels M (2018) Novel constructs-half-life extension, in *Challenges in Protein Product Development* (Warne NW, and Mahler H-C eds), pp 527-544 Springer International Publishing AG, Cham, Switzerland.

Sundy JS, Barag HSB, and Yood RA (2001) Efficacy and tolerability of pegloticase for the treatment of chronic gout in patients refractory to coventional treatment. *JAMA* **306**: 711-720.

Supersaxo A, Hein WR, and Steffan H (1990) Effect of molecular weight on the lymphatic absorption of water-soluble compounds following subcutaneous administration. *Pharm Res* **7**: 167-169.

Suzuki T, Ishii-Watabe A, Tada M, Kobayashi T, Kanayasu-Toyoda T, Kawanishi T, and Yamaguchi T (2010) Importance of neonatal FcR in regulating the serum half-life of therapeutic proteins containing the Fc domain of human IgG1: a comparative study of the affinity of monoclonal antibodies and Fc-fusion proteins to human neonatal FcR. *J Immunol* **184**: 1968-1976.

Swed A, Cordonnier T. Fleury F, and Boury F (2014) Protein encapsulation into PLGA nanoparticles by a novel phase separation method using non-toxic solvents. *J Nanomed Nanotechnol* **5**: 241.

Swierczewska M, Leec KC, and Leea S (2015) What is the future of PEGylated therapies? *Expert Opin Emerg Drugs* **20**: 531-536.

Tang L, Persky AM, Hochhaus G, and Meibohm B (2004) Pharmacokinetic aspects of biotechnology products. *J Pharm Sci* **93**: 2184-2204.

Taraghdari ZB, Imani R, and Mohabatpour F (2019) A review on bioengineering approaches to insulin delivery: a pharmaceutical and engineering perspective. *Macromol Biosci* doi: 10.1002/mabi.201800458.

Tibbitts J, Canter D, Graff R, Smith A, and Khawli LA (2016) Key factors influencing ADME properties of therapeutic proteins: A need for ADME characterization in drug discovery and development. *MAbs* 8: 229-245.

Tiede A (2015) Half-life extended factor VIII for the treatment of haemophilia A. *J Thromb Haemost*. **13 Suppl 1**: S176–S179.

Tijink BM, Laeremans T, Budde M, Stigter-van Walsum M, Dreier T, de Haard HJ, Leemans CR, and van Dongen GA. (2008) Improved tumor targeting of anti-epidermal growth

factor receptor Nanobodies through albumin binding: taking advantage of modular Nanobody technology. *Mol Cancer Ther* **7**: 2288-2297.

Trujillo JM, and Nuffer W (2014) Albiglutide: a new GLP-1 receptor agonist for the treatment of type 2 diabetes. *Ann Pharmacother* **48**: 1494-1501.

Unverdorben F, Hutt M, Seifert O, and Kontermann RE (2015) A Fab-selective immunoglobulin-binding domain from Streptococcal Protein G with improved half-life extension properties. *PLoS One* **10**: e0139838, 1-13.

Unverdorben F, Richter F, Hutt M1, Seifert O, Malinge P, Fischer N, and Kontermann RE. (2016) Pharmacokinetic properties of IgG and various Fc fusion proteins in mice. *MAbs* **8**: 120-128.

Van Roy M, Ververken C, Beirnaert E, Hoefman S, Kolkman J, Vierboom M, Breedveld E, 't Hart B, Poelmans S, Bontinck L, Hemeryck A, Jacobs S, Baumeister J, and Ulrichts H. (2015) The preclinical pharmacology of the high affinity anti-IL-6R Nanobody ALX-0061 supports its clinical development in rheumatoid arthritis. *Arthritis Res Ther* **17**: 135.

Vugmeyster Y, Xu X, Theil FP, Khawli LA, and Leach MW (2012) Pharmacokinetics and toxicology of therapeutic proteins: advances and challenges. *World J Biol Chem* **3**: 73-92.

Wang J, Cao H, Zhang JZH, and Qi Y (2018) Computational protein design with deep learning neural networks *Sci Rep* **8**: 6349, 1-9.

Wang M, Wang T, Guan Y, Wang F, and Zhu J (2019) The preparation and therapeutic roles of scFv-Fc antibody against Staphylococcus aureus infection to control bovine mastitis. *Appl Microbiol Biotechnol* **103**:1703-1712.

Wang W, Wang EQ, and Balthasar JP (2008) Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin Pharmacol Ther* **84**: 548-558.

Wang X, Ishida T, and Kiwada H (2007) Anti-PEG IgM elicited by injection of liposomes is involved in the enhanced blood clearance of a subsequent dose of PEGylated liposomes. *J Control Release* **119**: 236-244.

Wang Y, Shao J, Zaro JL, and Shen WC (2014b) Proinsulin-transferrin fusion protein as a novel long-acting insulin analog for the inhibition of hepatic glucose production. *Diabetes* **63**: 1779–1788.

Wang Y, Tian Z, Thirumalai D, and Zhang X (2014a) Neonatal Fc receptor (FcRn): a novel target for therapeutic antibodies and antibody engineering. *J Drug Target* **22**: 269-278.

Walsh G (2010) Biopharmaceutical benchmarks 2010. *Nat Biotechnol* **28**: 917-924.

Walsh G (2014) Biopharmaceutical benchmarks 2014. *Nat Biotechnol* **32**: 992-1000.

Walsh G (2018) Biopharmaceutical benchmarks 2018. *Nat Biotechnol* **36**: 1136-1145.

Ward ES and Ober RJ (2018) Targeting FcRn to generate antibody-based therapeutics. *Trends Pharmacol Sci* **39**: 892-904.

Wei J, Bera TK, Liu XF, Zhou Q, Onda M, Ho M, Tai CH, and Pastan I (2018) Recombinant immunotoxins with albumin-binding domains have long half-lives and high antitumor activity. *Proc Natl Acad Sci U S A* **115**:E3501-E3508.

Wu B, and Sun YN (2014) Pharmacokinetics of Peptide-Fc fusion proteins. *J Pharm Sci* **103**: 53-64.

Yuen KC, Conway GS, Popovic V, Merriam GR, Bailey T, Hamrahian AH, Biller BM, Kipnes M, Moore JA, Humphriss E, Bright GM, and Cleland JL (2013) A long-acting human growth hormone with delayed clearance (VRS-317): results of a double-blind, placebo-controlled, single ascending dose study in growth hormone-deficient adults. *J Clin Endrocrin Metab* **98**: 2595-2603.

Zaman R, Islam RA, Ibnat N, Othman I, Zaini A, Lee CY, and Chowdhury EH (2019) Current strategies in extending half-lives of therapeutic proteins. *J Control Release* doi: 10.1016/j.jconrel.2019.02.016.

Zhang F, Liu MR, and Wan HT (2014) Discussion about several potential drawbacks of PEGylated therapeutic proteins. *Biol Pharm Bull* **37**: 335-339.

Zhao H, Yao X, Xue C, Wang Y, Xiong X and Liu Z (2008) Increasing the homogeneity, stability and activity of human serum albumin and interferon-alpha2b fusion protein by linker engineering. *Protein Expr Purif* **61**: 73-77.

Zhou L, Wang HY, Tong S, Okamoto CT, Shen WC, and Zaro JL (2017) Single chain Fc-dimer-human growth hormone fusion protein for improved drug delivery. *Biomaterials* **117**: 24-31.

Zong Y, Tan X, Xiao J, Zhang X, Xia X, and Sun H (2019) Half-life extension of porcine interferon-α by fusion to the IgG-binding domain of streptococcal G protein. *Protein Expr Purif* **153**: 53-58.

Figure legends:

Fig. 1. Schematic depiction of FcRn-mediated recycling of chimeric proteins: Proteins chimerized with Fc portion of Ig or albumin is taken up by non-specific mechanisms into the cell. Once taken up into the cell, the chimeric proteins bind to FcRn at pH 6.0 in the endosome and are recycled back into the circulation where the chimeric proteins dissociate from the FcRn, as a result of low affinity, due to shift in pH to 7.4**.** Unfused proteins that do not bind to FcRn are degraded in the lysosome at pH 4.0-5.0 **(**Sockolosky and Szoka, 2015).

Fig. 2. Various approaches available to increase circulatory half-life of protein therapeutics.

Fig. 3. Various HLEPs available for generation of chimeric protein therapeutics (Strohl, 2015).

JPET Fast Forward. Published on April 22, 2019 as DOI: 10.1124/jpet.119.257063
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1: Examples of protein partners used in the generation of chimeric protein therapeutics with improved half-life.

HLEP	Effector protein (drug name)	Half-life with and without chimerization		Disease indication	Development Status	References
		Without	With			
Fc fusion	Factor IX (eftrenonacog-α)	~18 hrs	~57 hrs	Haemophilia	Approved by US-FDA in 2014	Shapiro et al., 2012; Strohl, 2015
Albumin fusion	GLP-1 (albiglutide)	1-2 mins	~4-7 days	Diabetes Mellitus	Approved by US-FDA in 2014	Kontermann, 2016; Strohl, 2015
Tf fusion	Proinsulin	0.5 hrs	>7 hrs	Diabetes Mellitus	Preclinical (BALB/c mice)	Wang et al., 2014b
XTENylation	Growth hormone (GH) (VRS-317 /somavaratan)	1.7 hrs	131 hrs (in adult)	GH deficiency	Phase 2 for adults (NCT02526420) Phase 3 for children (NCT0233	Fares et al., 2010; Moore et al., 2016; Strohl, 2015; Yuen et al., 2013

					9090)	
PASylation	Adenectin	49 mins	226 mins	Cancer	Preclinical (BALB/c mice)	Aghaabdollahian et al., 2019
ELPylation	GLP-1 (PB1023)	1-2 mins	~ 36 hrs	Diabetes Mellitus	Phase 2 (NCT01658501)	Strohl, 2015
CTP fusion	Growth hormone (GH) (MOD-4023)	1.7 hrs	~33-37 hrs	GH deficiency	Phase 3 (NCT02968004)	Fares et al., 2010; Hershkovitz et al., 2016; Strasburger et al., 2017

The NCT number represents the clinicaltrials.gov identifier for the clinical study of the respective drug molecule. Web addresses for information taken from clinicaltrials.gov (Accessed 02-04-2019):

https://clinicaltrials.gov/ct2/show/NCT02526420;

https://clinicaltrials.gov/ct2/show/NCT02339090;

https://clinicaltrials.gov/ct2/show/NCT01658501;

https://clinicaltrials.gov/ct2/show/NCT02968004.

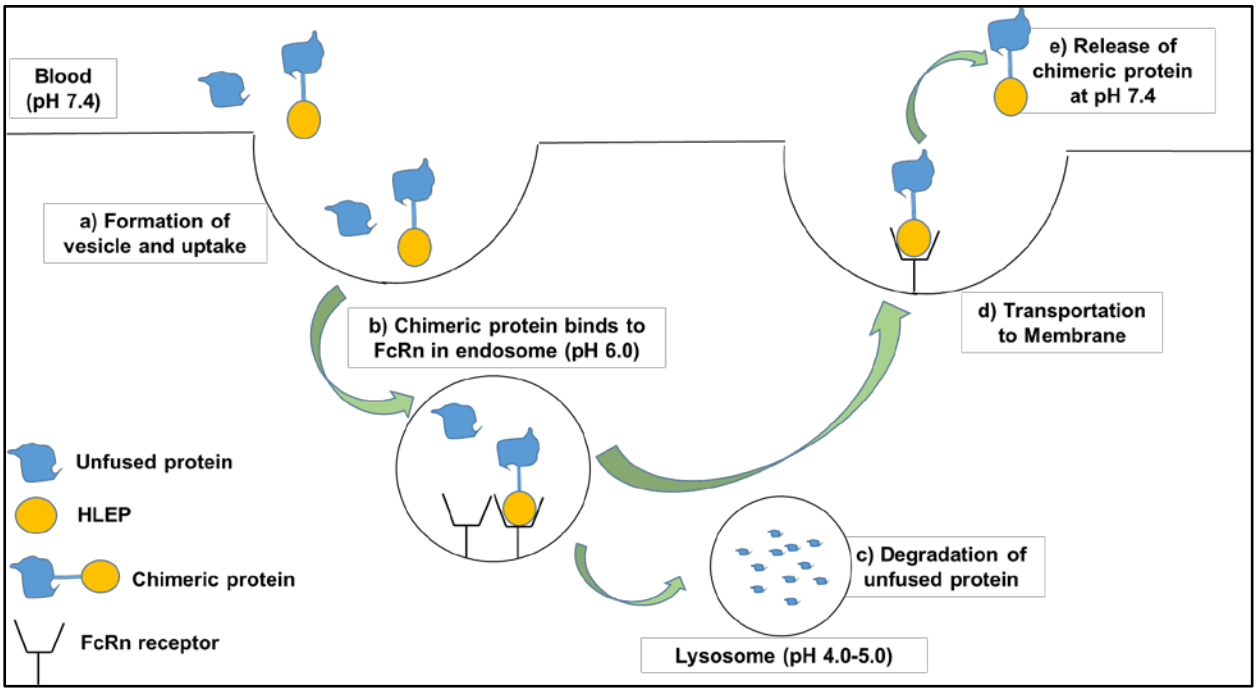
Table 2: Various linkers used for chimerization of proteins (Chen et al., 2013a; Chen et al., 2013b).

Type of Linker	Properties	Example
Flexible Linkers	➢ Composed of small polar/non polar amino acids. ➢ Used when domains require movement. ➢ Do not allow separation of operational domains/protein partners.	$(G_4S)_n$, $(G)_8$
Rigid Linkers	➢ Used when spatial distance between domains/protein partners is required. ➢ Freedom for domains to perform respective functions.	$(EAAAK)_n$, $(XP)_n$
Cleavable linkers	➢ Free functional protein partners released subsequent to cleavage *in vivo*. ➢ Cleaved in the presence of proteases or reducing agents.	VSQTSKLTRAETVFPDV, dithiocyclopeptide linker

JPET Fast Forward. Published on April 22, 2019 as DOI: 10.1124/jpet.119.257063
This article has not been copyedited and formatted. The final version may differ from this version.

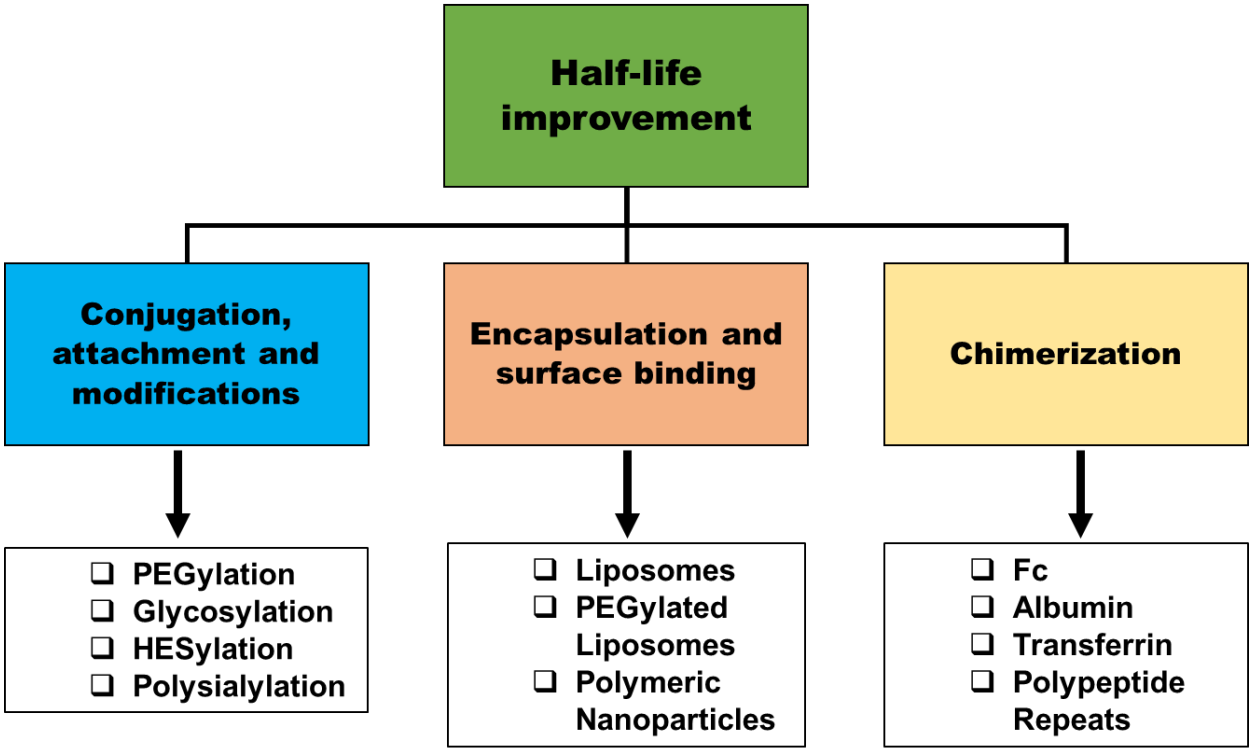
Figures:

Figure 1



JPET Fast Forward. Published on April 22, 2019 as DOI: 10.1124/jpet.119.257063
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



JPET Fast Forward. Published on April 22, 2019 as DOI: 10.1124/jpet.119.257063
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 3