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Strategies to improve plasma half life time of peptide and protein drugs

Review Article

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Summary. Due to the obvious advantages of long-acting peptide and protein drugs, strategies to prolong plasma half life time of such compounds are highly on demand. Short plasma half life times are commonly due to fast renal clearance as well as to enzymatic degradation occurring during systemic circulation. Modifications of the peptide/protein can lead to prolonged plasma half life times. By shortening the overall amino acid amount of somatostatin and replacing L-analogue amino acids with D-amino acids, plasma half life time of the derivate octreotide was 1.5 hours in comparison to only few minutes of somatostatin. A $PEG_{2,40\,K}$ conjugate of INF-α-2b exhibited a 330-fold prolonged plasma half life time compared to the native protein. It was the aim of this review to provide an overview of possible strategies to prolong plasma half life time such as modification of N- and C-terminus or PEGylation as well as methods to evaluate the effectiveness of drug modifications. Furthermore, fundamental data about most important proteolytic enzymes of human blood, liver and kidney as well as their cleavage specificity and inhibitors for them are provided in order to predict enzymatic cleavage of peptide and protein drugs during systemic circulation.

Keywords: Peptides – Proteins – Plasma half life time – N-C-terminus – PEGylation

Introduction

Due to the great progress in the field of recombinant technology and biotechnology, it is possible to produce a large number of peptides and proteins in commercial quantities. The majority of these drugs are administered via parenteral routes. Although it is possible to produce a multitude of peptide and protein based drug candidates, many drugs which exhibit promising pharmacological activities fail to show convincing effects *in vivo*. This is due to various reasons, including low stability or unexpected immunogenicity and toxicity. One main problem is that several therapeutic peptides and proteins exhibit a short plasma half life time. Peptides and proteins often display half life times in the range of few minutes to few hours. Half life times of only few minutes are in most cases not effective in order to deliver sufficient drug amounts to the target tissue. Short plasma half life times are commonly due to fast renal clearance which is connected to the hydrophilic properties of most of these agents as well as their often small size or to enzymatic degradation caused by enzymes occurring in blood, liver and kidney. Strategies to prolong plasma half life time may lead to improved pharmacokinetic profiles of established drugs and may even offer new indications for drug use. Moreover, prolongation of plasma half life time is often a prerequisite for numerous drug candidates for their clinical use at all.

A well known example for a targeted modification is octreotide, a drug which is used in the treatment of gastrointestinal tumors. It is a synthetic peptide based on the amino acid sequence of the endogen hormone somatostatin, which is a 14-peptide tetradecapeptide that inhibits pancreatic exocrine and endocrine secretion. Its clinical application has been limited by its short half life time of only few minutes. To overcome this drawback, octreotide was developed. By shortening the overall amino acid sequence of somatostatin from 14 to 8 and the replacement of L-amino acids by D-amino acids, the enzymatic stability was enhanced which consequently led to an improvement of plasma half life time from few minutes up to 1.5 hours (Harris, 1994).

Due to the fact that a broad variety of proteolytic enzymes occur in the human body, it is important to

Table 1. Tissue source of human peptidases/proteases

EC number	*Recommended name* and synonyms	Blood	Liver	Kidney
2.3.2.2	*Gamma-glutamyltransferase* gamma-glutamyl transpeptidase, gamma-GPT, gamma-GTase, GGT	X	X	X
3.1.2.12	*S-formylglutathione hydrolase* FGH, Ubiquitin thiolesterase 12		X	
3.3.2.6	*Leukotriene-A4 hydrolase* LTA-4 hydrolase		X	X
3.4.11.1	*Leucyl aminopeptidase* aminopeptidase I, cathepsin III, cytosol aminopeptidase, FTBL protein, peptidase S		X	X
3.4.11.2	*Membrane alanyl aminopeptidase* amino-oligopeptidase, aminopeptidase M, aminopeptidase N, aminopeptidase, microsomal, APN, CD13	X		
3.4.11.3	*Cystinyl aminopeptidase* alpha-Aminoacyl-peptide hydrolase, insulin-regulated aminopeptidase, IRAP, oxytocinase, P-LAP		X	X
3.4.11.4	*Tripeptide aminopeptidase* peptidase T			X
3.4.11.5	*Prolyl aminopeptidase* cytosol aminopeptidase V		X	
3.4.11.6	Aminopeptidase B L-RAP, leukocyte-derived arginine aminopeptidase		X	
3.4.11.7	*Glutamyl aminopeptidase* aminopeptidase A, angiotensinase A, glutamyl aminopeptidase	X		X
3.4.11.9	*X-Pro aminopeptidase* aminopeptidase P, AP-P	X		X
3.4.11.14	*Cytosol alanyl aminopeptidase* Aminopolypeptidase, tripeptidase		X	X
3.4.13.3	*X-His dipeptidase* aminoacyl-L-histidine hydrolase, carnosinase	X	X	X
3.4.13.9	*X-Pro dipeptidase* post-proline-cleaving aminopeptidase, prolidase, quiescent cell proline dipeptidase		X	X
3.4.13.18	*Cytosol nonspecific dipeptidase* DPP8, human cytosolic non-specific dipeptidase, prolyl dipeptidase	X	X	X
3.4.13.19	*Membrane dipeptidase* MBD, nonspecific dipeptidase			X
3.4.13.20	*Beta-Ala-His dipeptidase* serum carnosinase	X		
3.4.14.1	*Dipeptidyl-peptidase I* cathepsin C, cathepsin J, dipeptidyl aminopeptidase I			X
3.4.14.2	*Dipeptidyl-peptidase II* dipeptidyl peptidase, DP II, DPP-II	X		X
3.4.14.5	*Dipeptidyl-peptidase IV* (GLP1)-degrading enzyme, ADA binding protein, ADABP, adenosine deaminase binding protein, attractin, CD26, DP IV, glucagon-like peptide 1-degrading enzyme	X		X
3.4.15.1	*Peptidyl-dipeptidase A* ACE, angiotensin converting enzyme, peptidase P, peptidyl dipeptidase A	X	X	X
3.4.16.2	*Lysosomal Pro-X carboxypeptidase* endothelial cell prekallikrein activator, HUVEC PK activator, matrix PK activator, PKA, PRCP, prolylcarboxypeptidase, serine protease prolylcarboxypeptidase	X		X
3.4.17.2	*Carboxypeptidase B* HBCPB, PCB	X		
3.4.17.3	*Lysine carboxypeptidase* carboxypeptidase N, CPN, Plasma carboxypeptidase B	X	X	X
3.4.17.20	*Carboxypeptidase U* CPR, TAFI, thrombin-activatable fibrinolysis inhibitor	X	X	

(*continued*)

Table 1 (*continued*)

EC number	*Recommended name* and synonyms	Blood	Liver	Kidney
3.4.17.21	*Glutamate carboxypeptidase II* Acetylaspartylglutamate dipeptidase, N-Acetylated alpha-linked acidic dipeptidase, Prostrate-specific membrane antigen	X		
3.4.18.1	*Cathepsin X* cathepsin Z, cysteine-type carboxypeptidase		X	
3.4.19.3	*Pyroglutamyl-peptidase I* aminopeptidase, pyroglutamate, PGPEP1	X		X
3.4.19.9	*Gamma-glutamyl hydrolase* carboxypeptidase G, folic acid conjugase, polyglutamate hydrolase	X	X	
3.4.21.41	*Complement subcomponent C1r*	X	X	
3.4.21.42	*complement subcomponent C1s* protease C1s	X		
3.4.21.B1	*hyalorunan-binding serine protease* factor seven activating protease, FSAP, PHBP	X	X	X
3.4.21.B7	*mannan-binding lectin-associated serine protease 1* MASP-1, P100, RaRF	X	X	
3.4.21.B13	*protease do* HtrA			X
3.4.21.B39	*stratum corneum tryptic enzyme* K5, kallikrein protein 5, SCTE, strateum–corneum trypsin-like serine protease	X		X
3.4.22.16	*cathepsin H*		X	X
3.4.22.B13	*caspase-9* APAF, ICE-like apoptotic protease 6		X	
3.4.22.B40	*caspase-4* ICH-3 protease, TX protease		X	X
3.4.22.B41	*caspase-5* TY protease		X	
3.4.23.45	*memapsin 1* aspartic protease BACE2, protease ASP1			X
3.4.24.11	*Neprilysin* acute lymphoblastic leukemia antigen, common acute lymphoblastic leukemia antigen, CALLA, Endopeptidase-2, neutral metallendopeptidase	X		X
3.4.24.18	*meprin A* PPH alpha			X
3.4.24.80	*membrane-type matrix metalloproteinase-1*		X	
3.4.24.81	*ADAM10 endopeptidase* metalloproteinase MADM			X
3.4.24.86	*ADAM 17 endopeptidase* H-TACE, metalloprotease TACE, TNF-alpha processing protease	X		X
3.4.24.B9	*ADAM9 endopeptidase* cellular disintegrin-related protein, M12.209, MDC-9, meltrin gamma		X	
3.4.24.B13	*ADAMTS13 endopeptidase* M12.241, van Willebrand factor processing activity, vWF protease	X	X	X

identify the potential enzymes that degrade a certain peptide or protein during systemic circulation. Beside the knowledge regarding tissue localisation of various peptidases and proteases, their cleavage specificity is of particular interest. In the context of enzymatic degradation during systemic circulation, blood, liver and kidney are most important. In order to modify peptides and proteins in an appropriate way to enhance their stability and prolong their plasma half life time it is therefore necessary to know which enzymes cleave the drug and what cleavage specificity is exhibited by the particular proteolytic enzyme. Beside of proteolytic enzymes, several other types of drug-metabolizing enzymes (DME) occur within the human body such as Cytochrome P450 enzymes. Various reviews dealing with the topic of predicting drug metabolism and human pharmacokinetic parameters from *in vitro* as well as preclinical data are already published (Pelkonen et al., 2005; Obach et al., 1997; Chaturvedi et al., 2001).

It was therefore the aim of this review to provide an overview of the various peptidases and proteases occurring in human blood, liver and kidney and about their

cleavage specificity. Furthermore, methods to evaluate the protective effect of modifications to improve metabolic stability as well as strategies to prolong plasma half life time of peptides and proteins shall be discussed.

1. Systemic peptidases and proteases affecting plasma half life time

Referring to plasma half life time, most important compartments concerning enzymatic degradation of peptides and proteins are in first instance blood, liver and kidney. Orally administered peptides or proteins, which are absorbed from the stomach or the intestine, are transported in the venous blood via the vena portae through the liver to enter systemic circulation.

Parenteral administered drugs have to pass liver and kidney, which are with more than 1 liter blood/min organs which are very well supplied with blood. The liver as well as the kidney contains several proteolytic enzymes. In the kidneys, glomerular ultrafiltrate is pressed

Table 2. Aminopeptidases of human blood, liver and kidney

EC number	Specificity	Localization
3.3.2.6	bifunctional enzyme acting as an epoxid hydrolase and also as an aminopeptidase	Cytosol (erythrocyte, leukocyte, liver) (Samuelsson et al., 1989)
3.4.11.1	release of an N-terminal amino acid, Xaa-/-Yaa-, in which Xaa is preferably Leu, but may be other amino acids including Pro although not Arg or Lys, and Yaa may be Pro. Amino acid amides and methyl esters are also readily hydrolysed, but rates on arylamides are exceedingly low	Cytoplasm (liver) (Kohno et al., 1986)
3.4.11.2	release of an N-terminal amino acid, Xaa-/-Xbb- from a peptide, amide or arylamide. Xaa is preferably Ala, but may be most amino acids including Pro (slow action). When a terminal hydrophobic residue is followed by a prolyl residue, the two may be released as an intact Xaa-Pro dipeptide	Membrane (mucosal membrane) (McClellan et al., 1980)
3.4.11.3	release of an N-terminal amino acid, Cys-/-Xaa-, in which the half-cystine residue is involved in a disulphide loop, notably in oxytocin or vasopressin. Hydrolysis rates on a range of aminoacyl arylamides exceed that for the cystinyl derivative, however	Lysosome, mitochondrion, nucleus soluble (Lampelo et al., 1979), membrane (placenta, serum) (Matsumoto et al., 2000)
3.4.11.4	release of N-terminal residue from tripeptide	–
3.4.11.5	release of N-terminal proline from peptide	–
3.4.11.6	release of N-terminal Arg and Lys from oligopeptides when P1′ is not Pro, also acts on arylamides of Arg and Lys	Cytosol (leukocyte) (Mendz et al., 1989)
3.4.11.7	release of N-terminal glutamate (and to a lesser extend aspartate) from a peptide	Particle bound (intestine) (Sterchi et al., 1981)
3.4.11.9	release of any N-terminal amino acid, including proline, that is linked to proline, even from a dipeptide or tripeptide	Cytosol (leukocyte, platelet) (Cottrell et al., 2000), extracellular (plasma) (Adam et al., 2002)
3.4.11.14	release of N-terminal amino acid, preferentially alanine, from a wide range of peptides, amides and arylamides	–
3.4.14.1	release of an N-terminal dipeptide, Xaa-Xbb-/-Xcc, except when Xaa is Arg or Lys, or Xcc is Pro	Lysosome (kidney) (Cigic et al., 1999)
3.4.14.2	release of an N-terminal dipeptide, Xaa-Xbb-/-Xcc, preferentially when Xbb is Ala or Pro. Substrates are oligopeptides preferentially tripeptides	Lysosome (placenta) (Stoeckel-Maschek et al., 2000)
3.4.14.5	release of an N-terminal dipeptide, Xaa-Xbb-/-Xcc-, from a polypeptide, preferentially when Xbb is Pro, provided Xcc is neither Pro nor hydroxyproline	Extracllular (seminal plasma, serum), membrane (kidney) (Oefner, 2003)
3.4.19.3	release of an N-terminal pyroglutamyl group from a polypeptide, the second amino acid generally not being Pro	–
3.4.22.16	hydrolysis of proteins, acting as an aminopeptidase as well as an endopeptidase	–
3.4.24.86	narrow endopeptidase specificity. Cleaves Pro-Leu-Ala-Gln-AlaVal-Arg-Ser-Ser-Ser in the membrane-bound, 26-kDa form of tumor necrosis factor a (TNFa). Similarly cleaves other membrane-anchored, cell-surface proteins to "shed" the extracellular domains	Cytoplasm (fibroblast, leukocyte) (Reddy et al., 2000), membrane (kidney) (Schloendorff, 2000)

Table 3. Carboxypeptidases of human blood, liver and kidney

EC number	Specificity	Localization
3.4.15.1	release of a C-terminal dipeptide, oligopeptide-/-Xaa-Xbb, when Xaa is not Pro, and Xbb is neither Asp nor Glu. Thus, conversion of angiotensin I to angiotensin II, with increase in vasoconstrictor activity, but no action on angiotensin II	Membrane (kidney) (Takada et al., 1981)
3.4.16.2	cleavage of a Pro-/-Xaa bond to release a C-terminal amino acid	Lysosome (kidney), membrane (umbilical vein endothelial cell line) (Shariat-Madar et al., 2004)
3.4.17.2	preferential release of a C-terminal lysine or arginine amino acid	–
3.4.17.3	release of C-terminal basic amino acid, preferentially lysine	–
3.4.17.20	release of C-terminal Arg and Lys from a polypeptide	–
3.4.17.21	release of an unsubstituted, C-terminal glutamyl residue, typically from Ac-Asp-Glu or folylpoly-gamma-glutamates	Membrane (prostate gland) (Gregorakis et al., 1998)
3.4.18.1	release of C-terminal amino acid residues with broad specificity, but lacks action on C-terminal proline. Shows weak endopeptidase activity	–

out of the plasma. Molecules with a molecular mass below 5 kDa and which are not bound to plasma proteins can pass the filter completely, whereas for example only 1% of albumin with a molecular mass of 69 kDa can be found in the glomerular ultrafiltrate. Also secretive and absorbtive mechanisms play an important role.

Regarding enzymatic stability, it has to be distinguished between the localisation of the proteolytic enzymes in the particular tissue. Hydrophilic drugs such as peptides and proteins are rather degraded by soluble enzymes occurring in the blood and membrane bound enzymes than by enzymes that occur mainly or exclusively in the cytosoplasma. Although a broad variety of peptidases and proteases occur in the above mentioned tissues, it must be taken into consideration that most of them exhibit narrow cleavage specificity. In Table 1, an overview about peptidases and proteases occurring in human blood, liver and kidney is provided.

Exopeptidases can be divided into two groups, amino- and carboxypeptidases. Aminopeptidases cleave peptides at the N-terminal whereas carboxypeptidases cleave peptides at the C-terminal site. Several exopeptidases occuring in human blood, liver and plasma as well as there preferred cleavage sites are listed in Tables 2 and 3. Information concerning cleavage specificity of proteolytic enzymes was summarized from the enzyme database at http://www.brenda.uni-koeln.de. As mentioned above, the localization of peptidases and proteases in the particular tissue is important. Therefore information about tissue localization is also provided in Tables 2 and 3.

In Table 4, dipeptidases and endopeptidases of human blood, liver and kidney as well as their localization in the particular tissue are shown. Endopeptidases often exhibit narrow cleavage specificity. However, modification in order to stabilise peptide or protein drugs towards endopeptidases is commonly more challenging than protecting N- or C-terminal sites towards endopeptidase cleavage.

2. Methods to evaluate parameters important for peptide/protein drug plasma half life time

2.1. In vitro test models

A common method to evaluate peptide or protein stability towards systemic metabolism is to incubate the drug at 37 °C and pH 7.4 in serum, plasma or diluted plasma (Fredholt et al., 2000). Samples can be withdrawn at pre-determined time points in order to gain a concentration-time profile. Although this method gives a good overview about the stability enhancement efficacy of modifications in comparison to the unmodified drug, it has to be considered that some proteolytic enzymes occur exclusively in liver or kidney. To verify the influence of these organs regarding enzymatic degradation, homogenates of liver or kidney can be used for enzymatic stability studies (Powell et al., 1992; Boulanger et al., 1992). However, therapeutic peptides and proteins – which are commonly hydrophilic molecules – will be digested to a much higher extent by such homogenates, containing large amounts of cytosolic enzymes, than can be expected under *in vivo* conditions.

Another approach which leads to more detailed results is the incubation of the drug in a medium containing one or more isolated enzymes. In Fig. 1 for example, the time dependent degradation caused by purified porcine kidney dipeptidyl peptidase IV (DP IV) of glucose-dependent

Table 4. Peptidases/proteases of human blood, liver and kidney acting as dipeptidase or endopeptidase

EC number	Specificity	Localization
2.3.2.2.	5-L-glutamyl-peptide + an amino acid = peptide + 5-L-glutamyl amino acid	Extracellular (blood, duodenum) (Shaw, 1983), membrane (pancreas) [Sugimoto et al. (Characterization of gamma-GTP in a human pancreatic cancer cell line)]
3.1.2.12	S-formylglutathione + H_2O = glutathione + formate	Cytosol (liver) (Uotila et al., 1974)
3.4.13.3	hydrolysis of Xaa-/-His dipeptides	Cytosol
3.4.13.9	hydrolysis of Xaa-/-Pro dipeptides, also acts on aminoacyl-hydroxyproline analogs, no action on Pro-Pro	Extracellular, cytosol (JURKAT cell) (Chiravuri, 2000)
3.4.13.18	hydrolysis of dipeptides, preferentially hydrophobic dipeptides including prolyl amino acids	–
3.4.13.19	hydrolysis of dipeptides	Membrane (kidney) (Hooper et al., 1990)
3.4.13.20	preferential hydrolysis of the beta-Ala-/-His dipeptide (carnosine), and also anserine, Xaa-/-His dipeptides and other dipeptides including homocarnosine	–
3.4.19.9	hydrolysis of a gamma-glutamyl bond	Cytoplasm, lysosome, membrane (jejunum) (Gregory et al., 1987), soluble (intracellular, jejunum) (Reisenauer, 1977)
3.4.21.41	selective cleavage of Lys(or Arg)-/-Ile bond in complement subcomponent C1s to form the active form of C1s	–
3.4.21.42	cleavage of Arg-/-Ala bond in complement component C4 to form C4a and C4b, and Lys(or Arg)-/-Lys bond in complement component C2 to form C2a and C2b: the ''classical'' pathway C3 convertase	–
3.4.21.B1	endopeptidase activity. Cleaves C-terminal site of Lys and Arg	–
3.4.21.B7	endopeptidase activity. It triggers the activation of complement cascade by activating the C4 and C2 components. It activates the C4 component by cleaving the alpha-chain of C4	–
3.4.21.B13	endoprotease activity. Enzyme can degrade iciA, ada, casein and globin	–
3.4.21.B39	proteolytic cleavage of polypeptides	–
3.4.22.16	hydrolysis of proteins, acting as an aminopeptidase as well as an endopeptidase	–
3.4.22.B13	the preferred cleavage sequence is LEHD-/-. Binding of caspase-9 to Apaf-1 leads to activation of the protease which then cleaves and activates caspase-3. Proteolytically cleaves poly(ADP-ribose) polymerase (PARP)	Cytosol (JURKAT cell) (Reisenauer, 1977)
3.4.22.B40	the preferred cleavage sequence is WEHD-/- or LEHD-/-. Efficient cleavage of p35 and pro-caspase-3	–
3.4.22.B41	the preferred cleavage sequence is WEHD-/- or LEHD-/-. Caspase-5 has protease activity on its own precursor and cleaves p35 and pro-caspase-3	–
3.4.23.45	broad endopeptidase specificity. Cleaves Glu-Val-Asn-Leu-Asp-Ala-Glu-Phe in the Swedish variant of Alzheimer's amyloid precursor protein	Membrane (Fluhrer et al., 2002)
3.4.24.11	preferential cleavage of polypeptides between hydrophobic residues, particularly with Phe or Tyr at P1′	Membrane (cerebrospinal fluid, plasma) (Spillantini et al., 1990)
3.4.24.18	hydrolysis preferentially on carboxyl side of hydrophobic residues	Membrane (Yamaguchi et al., 1994)
3.4.24.80	endopeptidase activity. Activates progelatinase A by cleavage of the propeptide at Asn37-/-Leu. Other bonds hydrolysed include Gly35-/-Ile in the propeptide of collagenase 3, and Asn341-/-Phe, Asp441-/-Leu and Gln354-/-Thr in the aggrecan interglobular domain	Cytoplasm, membrane (liver) (Seiki, 2003)
3.4.24.81	endopeptidase of broad specificity	Membrane (kidney) (Anders et al., 2001)
3.4.24.86	narrow endopeptidase specificity. Cleaves Pro-Leu-Ala-Gln-AlaVal-Arg-Ser-Ser-Ser in the membrane-bound, 26-kDa form of tumor necrosis factor a (TNFa). Similarly cleaves other membrane-anchored, cell-surface proteins to ''shed'' the extracellular domains	Cytoplasm, membrane (kidney) (Schloendorff et al., 2000)
3.4.24.B9	proteolytic cleavage of proteins	Extracellular (liver) (Hotoda et al., 2002), membrane
3.4.24.B13	proteolytic cleavage of von Willebrand factor	Extracellular (plasma, serum) (Furlan et al., 1996)

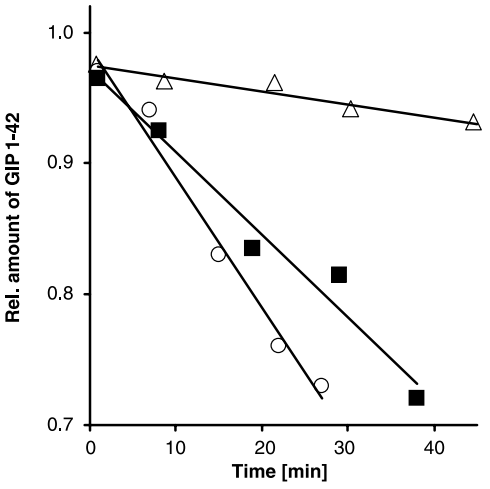

Fig. 1. Quantification of the DP IV-catalyzed GIP1–42 hydrolysis in the presence of specific DP IV inhibitors using MALDI-TOF MS; ○ represents substrate turnover in the absence of inhibitor, ■ represents turnover in the presence of alanine–thiazolidide and Δ represents turnover in the presence of isoleucine–thiazolidide; slightly modified from Pauly et al. (1996)

insulinotropic polypeptide (1–42) (GIP1–42) with and without inhibitors is shown (Pauly et al., 1996). Knowing the theoretical cleavage sites may allow a more specific alteration of the drug, especially when it is degraded by endopeptidases.

Needless to say that only a small amount of isolated proteolytic enzymes is commercially available and the isolation of certain enzymes can be intricate. Therefore another approach is the utilization of specific enzyme inhibitors. A reasonable proceeding is to first identify the theoretical eligible peptidases and proteases and then to systematically add specific inhibitors to the plasma in order to see if a stabilisation of the drug can be achieved. Thereafter, the drug can be modified in a well-directed manner. Various inhibitors of human peptidases and proteases as well as the molecular mass of the proteolytic enzymes are shown in Tables 5–7.

Renal excretion of most peptides and proteins is determined by glomerular filtration and reabsorption in the proximal tubule. Physico-chemical properties of the compound as well as its plasma protein binding ability can be used to predict renal clearance. Of course these parameters are less predictable where active secretion or reabsorption and saturation kinetics are involved.

If peptides and proteins are degraded by proteolytic enzymes, it is generally important to identify the frag-

Table 5. Inhibitors of human aminopeptidases

EC number	Molecular mass [kDa]	Inhibitors
3.3.2.6	49 (Ohishi et al., 1987)	14,15-Leukotriene A4, $HgCl_2$, Leukotriene A4, Leukotriene A4 methyl ester, N-Ethylmaleimide, PCMB (Ohishi et al., 1987)
3.4.11.1	326 (Kohno et al., 1986)	1,10-Phenanthroline, Amastatin, Bestatin, EDTA, Iodoacetic acid, N-Ethylmaleimide, p-Chloromercuribenzoate (Kohno et al., 1986)
3.4.11.2	240 (Tokioka-Terao et al., 1984)	1,10-Phenantroline (McClellan et al., 1980), 8-Hydroxychinoline (McClellan et al., 1980), Bacitracin (Yamamoto et al., 1994; Langguth et al., 1994), Bestatin (Okagawa et al., 1994), EDTA (Yamamoto et al., 1994), Puromycin (Langguth et al., 1994)
3.4.11.3	340 (Lampelo et al., 1979)	1,10-Phenanthroline, Co^{2+}, EDTA, EGTA, L-Methionine, Ni^{2+}, Zn^{2+} (Lampelo et al., 1979)
3.4.11.4	100 (Lees et al., 1990)	Cd^{2+}, Cu^{2+}, Hg^{2+}, N-Ethylmaleimide, p-Hydroxymercuriphenyl sulfonate, Zn^{2+} (Lees et al., 1990)
3.4.11.5	300 (Matsushima et al., 1991)	–
3.4.11.6	220 (Nagata et al., 1991)	Arphamenine A, Arphamenine B, Bestatin, Cd^{2+}, Cu^{2+}, Hg^{2+}, p-Chloromercuribenzoate (Nagata et al., 1991)
3.4.11.7	190 (Nagatsu et al., 1970)	1,10-Phenantroline, EDTA, Puromycin (Auricchio et al., 1978)
3.4.11.9	140 (Cottrell et al., 2000)	1,10-Phenanthroline, apstatin, Ca^{2+}, Co^{2+}, Cu^{2+}, Dithiothreitol, EDTA, Glutathione, Mg^{2+}, Ni^{2+}, Zn^{2+} (Cottrell et al., 2000)
3.4.11.14	223, 242	Benzylpenicillenic acid, Penicillin, Puromycin (Little et al., 1976)
3.4.14.1	210 (Mantle, 1991)	Leupeptin, NEM, p-Hydroxymercuriphenyl sulfonate (Mantle, 1991)
3.4.14.2	–	Bestatin, Diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride, Tris (Sakai et al., 1987)
3.4.14.5	230–250 (Shibuya-Saruta et al., 1996)	Cd^{2+}, Diprotin A, Hg^{2+}, Leu, Lys, Met, PCMB, $SrCl_2$, Zn^{2+} (Shibuya-Saruta et al., 1996), Ala-thiazolidide, Ile-thiazolidide (Pauly et al., 1999, 1996), NVP DPP728 (Ahren et al., 2002), valine-pyrrolidide (Deacon et al., 1998)
3.4.19.3	22 (Mantle et al., 1991)	Amastatin, Chymostatin, Elastinal, Leupeptin (Mantle et al., 1991)
3.4.22.16	28 (Schwartz et al., 1980)	chymostatin B, Iodoacetamide, Leupeptin, Puromycin, Soybean trypsin inhibitor (Schwartz et al., 1980)
3.4.24.86	120 (Schloendorff et al., 2000)	1,10-Phenanthroline, BB 94, EndoH, Hydroxamate, TACE-pro domain

Table 6. Inhibitors of human carboxypeptidases

EC number	Molecular mass [kDa]	Inhibitors
3.4.15.1	155 (Stewart et al., 1981)	Captopril, EDTA, snake venom peptide (Stewart et al., 1981)
3.4.16.2	115 (Odya et al., 1981)	Diisopropyl fluorophosphate, Pepstatin, phenylmethylsulfonyl fluoride (Odya et al., 1981)
3.4.17.2	32–33 (Marinkovic et al., 1977)	6-Amino-n-hexanoic acid, Co^{2+}, EDTA, SDS, Urea
3.4.17.3	280 (Schweisfurth, 1984)	2,3-dimercatopropan-1-ol, 2-Mercaptoethanol, 6-Aminohexanoic acid, Cd^{2+}, $CoCl_2$, EDTA, Hg^{2+}, $MnCl_2$, $NiSO_4$, Zn^{2+} (Schweisfurth, 1984)
3.4.17.20	435 (Wang et al., 1994)	1,10-Phenanthroline, 2-Guanidinoethylmercaptosuccinic acid (Wang et al., 1994), 2-Mercaptoethanol, 2-mercaptomethyl-3-guanidino-ethylthiopropanoic acid, Dithiothreitol, EDTA, Epsilon-aminocaproic acid, guanidinoethyl-mercaptosuccinic acid, p-Chloromercuribenzoate, peptide inhibitor from Hirudo medicinalis, Potato carboxypeptidase inhibitor (Bouma et al., 2001)
3.4.17.21	84 (Carter et al., 1996)	N-Acetyl-beta-L-Asp-L-Glu, Quisqualic acid (Carter et al., 1996),
3.4.18.1	–	CA-074, Chicken cystatin, cystatin C, GFG-semicarbazone, Stefin A (Klemencic et al., 2000)

ments because they can exhibit pharmacological activity. It is for example well known that the terminal located helices of teriparatide are essential for bioactivity which is mediated by an activation of cAMP/protein kinase-A (PKA) as well as protein kinase-C (PKC). C-truncated derivatives such as PTH 1–31 are able to stimulate intracellular cAMP accumulation. However, it has been demonstrated that PTH 1–31 is less potent to increase serum calcium levels in mice in comparison to PTH 1–34 (Mohan et al., 2000). Fujimori et al. demonstrated that activation of cAMP/PKA system requires the N-terminal amino acids 1 and 2 whereas the phospholipase-C/PKC system is coupled to a longer domain of the hormone's N-terminus (Fujimori et al., 1992). Also Tsomaia et al. showed that the N-terminal residues (1–4) of the signalling domain plays a significant role in PTH action (Tsomaia et al., 2004). Furthermore it has been demonstrated, that the truncated fragment PTH 2–34 was only 67% as potent as PTH 1–34 and deletion of the first two amino acids at the N terminus abolished the hormone's ability to stimulate cAMP production in UMR-106-01 cells (Civitelli et al., 1994). Moreover, it was shown that also the PTH analogues 3–34, 7–34 and 13–34 did not stimulate cAMP production (Yu et al., 1997).

Fragments of enzymatic peptide/protein drug hydrolysis can be isolated by HPLC and identified by mass spectroscopy (Hernandez-Ledesma et al., 2005). Knowing the cleavage sites of a peptide/protein drug allows a well-directed modification leading to improved enzymatic stability. Also simple methods such as thin layer chromatography can be helpful, especially for the identification of single amino acids cleaved by amino- or carboxypeptidases (Werle et al., 2006).

2.2. In vivo test models

Results of *in vitro* studies lead to specific modification of the drug in order to enhance its enzymatic stability. The ultimate proof of prolonged plasma half life time however, has to be provided by *in vivo* studies. Above all, *in vivo* studies are necessary for systems which prolong plasma half life time only partly by improving enzymatic stability such as PEGylation or oligomerization. After injection, blood samples are withdrawn at pre-determined time points and the degradation process is stopped for example by adding inhibitors such as EDTA or trifluoro acetic acid to gain a concentration-time curve. In Fig. 2 the time dependent plasma concentration of intravenously administered IFN-α2b (I) in comparison to a PEGylated derivative (II) is shown. Peptide drugs and proteins can usually be evaluated by HPLC, RIA or ELISA (Song et al., 2002; Ziegler et al., 1984; Kekow et al., 1988).

Beside direct detection of the compound itself it is sometimes possible to monitor the biological response caused by the drug such as a decrease in blood glucose after insulin administration (Krauland et al., 2004).

To predict renal clearance for humans, the use of interspecies allometric scaling approaches has been quite successful (Dedrick, 1973; Boxenbaum, 1982; Sawada et al., 1984). Nevertheless, the practical value of this approach is strongly limited due to the fact that it requires experimentation in four to five species (Lin, 1998). Another and more simple approach for predicting human renal clearance is to use the ratio of glomerular filtration rate (GFR) between rats and humans (Lin, 1995). Ratios of renal clearance for various drugs in rats and humans are roughly similar to the ratio of GFR between these two species.

Table 7. Inhibitors of human di- and endopeptidases

EC number	Molecular mass [kDa]	Inhibitors
2.3.2.2.	300 (Echetebu et al., 1982)	Glutathione (Miller et al., 1976), glycine (Wahlefeld et al., 1983), maleate (Miller, et al., 1976), NH_4 (Miller et al., 1976), urea (Ikeda et al., 1995)
3.1.2.12	52, 58 (Uotila et al., 1974)	Ascorbate, $CaCl_2$, Co^{2+}, $CuSO_4$, Folate, Glutathione, $HgCl_2$, Iodoacetate, p-Hydroxymercuribenzoate, Zn^{2+} (Uotila et al., 1974)
3.4.13.3	160 (Lenney et al., 1982)	1,10-Phenanthroline, Co^{2+}, Dithiothreitol, Homocarnosine (Lenney et al., 1982) EDTA (Lenney et al., 1985)
3.4.13.9	108, 185 (Ohhashi et al., 1990)	Daunorubicin, Doxorubicin (Muszynska et al., 2001), p-Chloromercuribenzoate (Endo et al., 1989)
3.4.13.18	63, 85 (Masuda et al., 1994)	Cd^{2+}, Cu^{2+}, EDTA, Mn^{2+}, Ni^{2+}, p-Chloromercuribenzoate, Pb^{2+}, Zn^{2+}
3.4.13.19	135, 200 (Sugiura et al., 1978)	1,10-Phenanthroline, Co^{2+}, Cysteine, EDTA, N-Bromosuccinimide (Sugiura et al., 1978)
3.4.13.20	160 (Lenney et al., 1982)	–
3.4.19.9	110 (Lavoie et al., 1975)	Gamma-Diglutamate, p-Aminobenzoylglutamate, Pterine, pteroyldiglutamate, Zn^{2+} (Wang et al., 1986)
3.4.21.41	17 (Sim et al., 1977)	4-Nitrophenyl-4-guanidinobenzoate, C1bar-inhibitor, Diisopropyl fluorophosphate, Leupeptin, NaCl (Sim, 1981)
3.4.21.42	113 (Sumi et al., 1974)	3,4-Dichloroisocoumarin, 4-chloro-3-(3-isothiureidopropoxy)isocoumarin, 4-chloro-3-ethoxy-7-guanidinoisocoumarin, 4-chloro-7-guanidino-3-(2-phenylethoxy)isocoumarin, 4-chloro-7-guanidino-3-methoxyisocoumarin, 7-amino-4-chloro-3-(3-isothiureidopropoxy)isocoumarin (Kam et al., 1992)
3.4.21.B1	70 (Hunfeld et al., 1999)	alpha1-proteinase, alpha2-antiplasmin, Aprotinin, C1-esterase inhibitor, Co^{2+}, Cu^{2+} (Hunfeld et al., 1999)
3.4.21.B7	85 (Wong et al., 1999)	alpha-2-Macroglobulin, C1-inhibitor, PefablocSC (Wong et al., 1999)
3.4.21.B13	–	–
3.4.21.B39	40 (Yousef et al., 2003)	Alpha1-antitrypsin, alpha2-Macroglobulin (Yousef et al., 2003)
3.4.22.16	28 (Schwartz et al., 1980)	chymostatin B, Iodoacetamide, Leupeptin, Puromycin, Soybean trypsin inhibitor (Schwartz et al., 1980)
3.4.22.B13	–	acetyl-AEVD-CHO, acetyl-DEVD-CHO, acetyl-IETD-CHO, acetyl-WEHD-CHO, acetyl-YVAD-CHO, benzyloxycarbonyl-VAD-fluoromethylketone, cowpox serpin CrmA (Garcia-Calvo et al., 1998)
3.4.22.B40	–	acetyl-AEVD-aldehyde, acetyl-DEVD-aldehyde, acetyl-IETD-aldehyde, acetyl-WEHD-aldehyde, acetyl-YVAD-aldehyde, benzyloxycarbonyl-VAD-fluoromethylketone (Garcia-Calvo et al., 1998)
3.4.22.B41	–	acetyl-AEVD-aldehyde, acetyl-DEVD-aldehyde, acetyl-IETD-aldehyde, acetyl-WEHD-aldehyde, acetyl-YVAD-aldehyde, benzyloxycarbonyl-VAD-fluoromethylketone, cowpox serpin CrmA (Garcia-Calvo et al., 1998)
3.4.23.45	62, 65 (Fluhrer et al., 2002)	Cu^{2+}, Zn^{2+} (Kim et al., 2002)
3.4.24.11	–	Leu5-enkephalin, Met5-enkephalin, Phosphoramidon, Thiorphan, thiorphan-NH_2 (Spillantini et al., 1990)
3.4.24.18	200 (Sterchi et al., 1988)	1,10-Phenanthroline, Ca^{2+}, Captopril, DTT, EDTA, Zn^{2+} (Sterchi et al., 1988)
3.4.24.80	–	Marimastat, TIMP-2, TIMP-4 (Toth et al., 2002)
3.4.24.81	68 (Colciaghi et al., 2002)	BB3103, o-Phenanthroline, TAPI (Vincent et al., 2001)
3.4.24.86	120 (Schloendorff et al., 2000)	1,10-Phenanthroline, BB 94, EndoH, Hydroxamate, TACE-pro domain
3.4.24.B9	–	Aprotinin, Benzamidine, Hydroxamates, o-Phenanthroline, phosporamidon, TIMP-1, TIMP-3 (Amour et al., 2002)
3.4.24.B13	300 (Furlan et al., 1996)	Citrate, EDTA, EGTA, N-Ethylmaleimide, Z-Phe-Phe-CHN_2 (Furlan et al., 1996)

Therefore, knowledge of renal clearance in rats allows the approximate estimation of human renal clearance using GFR ratios.

3. Strategies to improve peptide/protein drug plasma half life time

Although several strategies to improve peptide/protein drug plasma half life are already established, drug modifications shall be based on an exact knowledge of the enzymatic susceptibility of the particular drug.

It was for example demonstrated, that dermorphin analogues with additional D-amino acid substitutions were cleaved more rapidly than the parent peptides. This may be due to remote secondary structural features which may be important for differential enzyme susceptibility (Darlak et al., 1988). In addition, studies of Rafferty et al. demonstrated that D-amino acid-substituted ana-

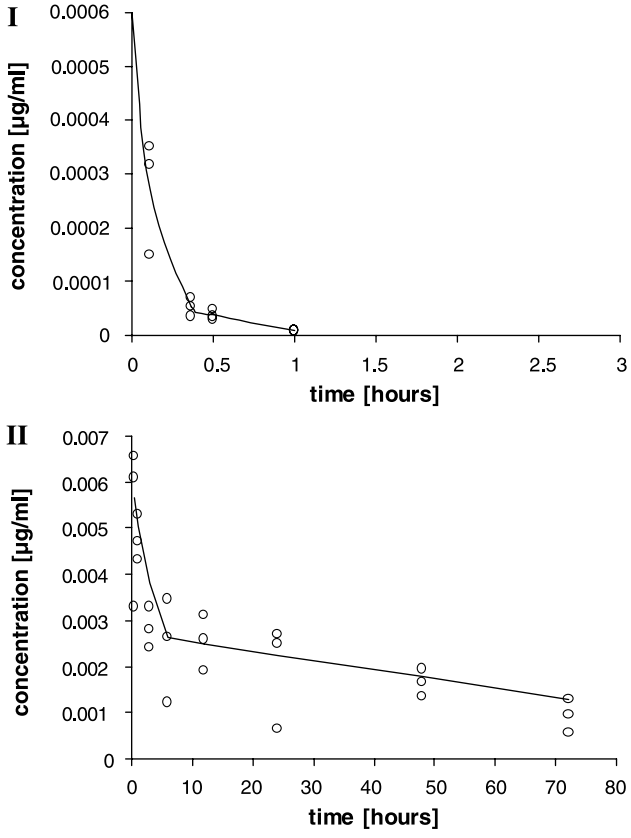

Fig. 2. IFN-α2b time course following a single 125 µg/kg intravenous bolus dose in male Sprague-Dawley rats. The symbols (open circles) represent the observed concentration data (three points per sampling time) from each treatment group (i.e., graph I for the unconjugated IFN; graph II for the $PEG_{2,40\,K}$-IFN-α2b). The continued lines represent the predicted values according to the best fitted-curve using compartment modelling; slightly modified from Ramon et al. (2005)

logues of growth hormone-releasing factor 1–29-amide did not exhibit significant different plasma half life times after intravenous injection in rats in comparison to the unmodified compound (Rafferty et al., 1988). These two examples emphasize the importance of well directed drug modification.

3.1. Modification of N- and C-terminus

As shown in Tables 1–3, several proteolytic enzymes occurring in plasma, liver and kidney which affect therapeutic peptides and proteins are exopeptidases. Therefore, a modification of either or both of the peptide/protein drug termini can in many cases significantly increase enzymatic stability. However, modifications always can lead to a loss of drug activity. One common way of terminal modification is N-acetylation and C-amidation. Brinckerhoff et al. for example significantly prolonged plasma stability of the immunogenic peptide MART-I_{27-35} by C-terminal amidation and/or N-terminal acetylation (Brinckerhoff et al., 1999). Also N-pyroglutamylation led to an improved enzymatic stability of glucagon-like-peptide-1_{7-36} (Green et al., 2004).

Various fatty acids of chain lengths ranging from 4 to 18 were conjugated to RC-160, a somatostatin analogue with anti-proliferative activity. The novel compounds exhibited greater resistance towards trypsin and serum degradation in comparison to unmodified RC-160 (Dasgupta et al., 2002).

Another approach is the specific covalent attachment of PEG to either or both termini of a peptide or a protein drug. The N-terminal modification of glucose-dependent insulinotropic polypeptide (GIP1–30) with 40 kDa PEG abrogated functional activity, whereas C-terminal PEGylation of GIP1–30 maintained full agonism at the GIP receptor and conferred a high level of dipeptidyl peptidase IV (DP IV) resistance. Moreover, the dual modification of N-terminal palmitoyl and C-terminal PEGylation resulted in a full agonist of comparable potency to native GIP that was stable to DP IV cleavage (Salhanick et al., 2005). Also Irwin et al. developed palmitate-derivitized analogues of N-terminal pyroglutamyl GIP which were completely resistant to DP IV degradation (Irwin et al., 2005).

In general, also a head-to-tail cyclization of peptides and proteins by the formation of an amide bond between C- and N-terminus in order to prevent exopeptidase caused degradation is possible. Of course, an alteration of the shape may lead to a loss in activity. Marastoni et al. prepared linear and head to tail cyclic hexapeptide analogues of peptide T. All compounds showed significant bioactivity and the cyclic peptides also proved to be highly resistant to degradation by plasma and brain enzymes (Marastoni et al., 1994).

3.2. Replacement of labile amino acids

A replacement of amino acids which are known to be susceptible for enzymatic cleavage is another strategy to delay degradation and therefore to improve plasma half life time. The substitution of L-amino acids with D-amino acids at both termini led to a stabilisation of several peptides (Powell et al., 1993). Various luteinizing hormone releasing hormone (LHRH) analogues which are substituted in positions 6, 10 or both and which furthermore are much more active and possess prolonged activity in comparison to LHRH are on the market including [D-Trp^6]LHRH (triptorelin), [D-Leu^6, Pro^9-NHEt]LHRH (leuprolide), [D-Ser(But)^6, Pro^9-NHEt]LHRH (buserelin),

[D-Ser(But)^6, Aza-Gly^{10}]LHRH (goserelin) and [D-Nal(2)^6]LHRH (nafarelin) (Holland et al., 1996).

Cetrorelix, a decapeptide, has a highly modified LHRH sequence, comprising 10 amino acids, five out of which are in a non-natural D-configuration. It is an antagonist of the luteinizing hormone releasing hormone (LHRH) and is highly resistant to degrading enzymes, e.g. chymotrypsin, pronase and nargase, for up to 50 h at 37 °C (Reissmann et al., 1994) which is in sharp contrast to several LHRH agonists which face almost complete degradation within few hours. The proteolytic stability of Cetrorelix is underlined in comparison with a diastereomeric analogue comprising L-configured citrulline in position 6 instead of D-citrulline as in Cetrorelix. This analogue is highly sensitive to degradation (Pinski et al., 1995).

Also analogues of glucagon-like peptide 1 (GLP-1), which were N-terminally substituted with threonine, glycine, serine or alpha-aminoisobutyric acid were more resistant to dipeptidyl peptidase IV than the unmodified compound (Deacon et al., 1998). By substituting the theoretical chymotrypsin cleavage site of a cystine-knot microprotein, stabilisation towards this peptidase was achieved (Werle et al., 2006). Marx et al. expressed, purified and characterised two chimeras of activated thrombin-activable fibrinolysis inhibitor (TAFIa), in which the non conserved residues were replaced by residues of pancreatic carboxypeptidase B. TAFIa is a labile carboxypeptidase B which is inactivated by conformational instability and proteolysis, whereas pancreatic carboxypeptidase B is a stable protease. One of the mutants displayed a markedly prolonged half life time (Marx et al., 2004). Also the GLP-1 analog [Ser]GLP-1 (7–36) amide, in which the second N-terminal amino acid alanine was replaced by serine was not significantly degraded by human and rat plasma in comparison to unmodified GLP-1 (Ritzel et al., 1998).

Strausberg et al. improved activity and stability of subtilisin by sequentially randomizing 12 amino acid positions in calcium-free subtilisin. The optimal amino acid for each randomized site was chosen based on stability and catalytic properties and became the parent clone for the next round of mutagenesis. Taken together, the 12 selected mutations increased subtilisin half-life at elevated temperature 15,000-fold (Strausberg et al., 2005).

3.3. Cyclization

Cyclization of a peptide or protein is a method to decrease proteolytic degradation and to prolong half life time. Growth regulating factor (GRF) and analogues were incubated in porcine plasma at 37 °C. GRF(1–29)-NH_2 displayed a half life time of only 13 minutes. Substitution of Gly15 by Ala15 only slightly prolonged plasma half life time (17 min), whereas side-chain to side-chain cyclization between Asp8 and Lys12 amino acid residues significantly improved the stability of GRF in plasma with $t1/2$ greater than 2 h. In addition, cyclization between Lys21 and Asp25 also improved GRF stability in plasma. Enzymatic stability and half life time were even more improved by substitution of D-Ala2 for Ala2 in the cyclic analogue (Su et al., 1991). Osapay et al. synthesised a series of cyclic somatostatin analogues containing a lanthionine bridge. The enzymatic stability of lanthionine–sandostatin and sandostatin was studied in rat brain homogenates. Although both compounds exhibited high enzymatic stability, the cyclic lanthionine–sandostatin had a 2.4-fold prolonged half life time in comparison to sandostatin (Osapay et al., 1997). Also a cyclic disulfide bonded analogue of indolicidin displayed greatly increased protease stability and the half life time in the presence of trypsin was increased 4.5-fold from 4 to 18 minutes (Rozek et al., 2003). Beside of cyclization in the molecule also a head to tail cyclization as mentioned in Section 3.1 can improve enzymatic stability.

3.4. Enzyme inhibition

An interesting approach for prolonging half life time of peptides and proteins might be the co-administration of specific enzyme inhibitors. It already has been demonstrated *in vitro* and *in vivo* that co-administration of enzyme inhibitors to oral dosage forms increases oral bioavailability (Fujii et al., 1985; Langguth et al., 1994; Morishita et al., 1992; Yamamoto et al., 1994). Recently, Pauly et al. (1990) showed the effectiveness of Ile-thiazolidide, a specific dipeptidyl dipeptidase IV inhibitor to increase circulating half life time of GLP-17–36. HPLC analysis of plasma following *in vivo* administration of 125I-labeled peptides showed that inhibition of DP IV by about 70% prevented the degradation of 90.0% of injected 125I-GLP-17–36 within 5 min, whereas only 13.4% remained unhydrolyzed in rats not treated with the DP IV-inhibitor after only 2 min (Pauly et al., 1999). Also the co-administration of the inhibitors NVP DPP728 and valine-pyrrolidide resulted in prolonged plasma half life time of the intact peptide (Ahren et al., 2002; Deacon et al., 1998). Wiedeman and Trevillyan summarized recent advances in the design of potent and selective small molecule inhibitors of DP IV and the potential challenges to the development of DP IV inhibitors for the

treatment of impaired glucose tolerance and type-2 diabetes (Wiedeman et al., 2003).

3.5. Increasing molecular mass: PEGylation and oligomerization

As a general rule, substances with a molecular mass below 5 kDa which are not bound to plasma proteins are completely excreted via the renal route, whereas molecules with a molecular mass over 50 kDa cannot or only in very small amounts be found in the glomerular ultrafiltrate. Accordingly, a main reason for short peptide and protein half life time beside enzymatic degradation is their fast renal excretion. Therefore, half life time can be prolonged by increasing drug size. Furthermore, a synergistic effect may be given by additional enzyme inhibition. Beside chemical modification of N- and C-termini which is usually an effective way to inhibit exopeptidases and replacement of labile amino acids, PEGylation offers the possibility to specifically protect endangered termini and furthermore increases molecular mass. Moreover, it is believed that PEGylation within the drug molecule leads to improved enzymatic stability mediated by a steric hindrance of proteolytic enzymes. Examples, where improved stability towards proteolytic digestion after PEGylation of the native protein was achieved are tumor necrosis factor, epidermal growth factor and interferon-α-2b (INF-α2b) (Li et al., 2002; Lee et al., 2003; Ramon et al., 2005). The stability of INF-α2b towards trypsin caused degradation was strongly improved by the conjugation of $PEG_{2,40\,K}$ to the native protein (Ramon et al., 2005). Furthermore, it has already been demonstrated that the effect of protecting PEGylated proteins from proteolysis is especially strong when high-molecular-weight, branched PEGs are used (Monfardini et al., 1995).

Poly(ethyleneglycol) (PEG) exhibits several properties that are of relevance for pharmaceutical applications: high water solubility, high mobility in solution, lack of toxicity and immunogenicity and ready clearance from the body (Delgado et al., 1992; Harris et al., 1997). Interestingly, many of these properties are transferred to PEG-protein or PEG-peptide conjugates. The extent of these feature are dependent on the molecular weight of the attached PEG. As demonstrated for example by He et al., only minor changes in immunogenicity of trichosanthin after modification with $PEG_{5\,k}$ was observed, whereas modification with $PEG_{20\,k}$ led to significantly reduced immunogenicity (He et al., 1999). Up to now, there are lots of data about the chemistry of PEGylation published (Kozlowski et al., 2001). Also a multitude of scientific articles have already been published where the effectiveness of PEG to improve half life time was clearly demonstrated (Sun et al., 2003). Lee et al. for example showed that site specific mono-PEGylation of GLP-1 led to a 16-fold increase in plasma half life time in rats (Lee et al., 2005). Moreover, some PEGylated proteins for human use are already on the market including PEGylated adenosine deaminase and asparaginase as well as α-Interferon. PEG-Intron, a PEGylated α-interferon marketed by Schering-Plough and approved by the FDA in 2001 for the treatment of hepatitis C, has an elimination half life time of 50 hours in comparison to 5 hours of the native α-interferon. Pegasys, a PEGylated α-interferon developed by Roche even exhibits an elimination half life time of 77 hours. Furthermore it has been shown recently by Ramon et al. that a $PEG_{2,40\,K}$ conjugate of INF-α-2b exhibited a 330-fold prolonged plasma half life time in rats compared to the native protein (Fig. 2) (Ramon et al., 2005).

Polymers of N-acetylneuraminic acid (polysialic acids) are naturally occurring, biodegradable, highly hydrophilic compounds which have no known receptors in the human body. After intravenous injection, polysialic acids exhibit long half-lives in the blood circulation and can therefore be used as carriers of short-lived drugs and small peptides. Furthermore, polysialic acids can be used in order to increase the circulatory half-life of proteins and thus serve as an alternative to the nonbiodegradable monomethoxy-poly(ethylene glycol) (Gregoriadis et al., 2000). Sialyation has been shown to effectively improve enzymatic stability of proteins as well as plasma half life time. Sialyated catalase for example was shown to be much more stable in the presence of specific endoproteases compared to the native enzyme (Fernandes et al., 1996). *In vivo* studies with polysialylated asparaginase in mice also revealed that beside of improved plasma stability also the half life time could be increased. Such an increase was greatest for the construct with the highest polysialylation (Fernandes et al., 1997).

PEGylation and sialyation prolong half life time by a combination of two mechanisms – improvement of enzymatic stability and decrease of renal excretion by increasing molecular mass – whereas oligomerization leads to prolonged half life time in first instance by increasing molecular mass. It was demonstrated for example, that a dimeric human erythropoietin, which was synthesized by chemical crosslinking of the monomeric form exhibited an increased plasma half life time in rabbits of more than 24 h compared to 4 h of the monomeric form. This effect is believed to be due to a decrease in glomerular filtration.

Furthermore, the dimer exhibited a more than 26-fold higher activity *in vivo* (Sytkowski et al., 1998).

3.6. Sustained delivery systems

Prolonged plasma half life time can also be achieved without chemical modification of the drug by the utilization of sustained delivery systems. Liposomes, for example, are widely used as drug carriers. As demonstrated by Kim et al., half life time of streptokinase incorporated in liposomes increased 16.3- and 6.1-fold, respectively in comparison to those of streptokinase alone after femoral administration in rats (Kim et al., 1998). Also intraperitoneal administered liposomes containing insulin exhibited a prolonged plasma half life time of 4–5 hours in contrast to free insulin solution in diabetic rats (Khaksa et al., 2000). In a pharmacokinetic *in vivo* study, after subcutaneous injections in rats, insulin like growth factor-1 (IGF-I) levels were sustained for 5–7 days with a multivesicular liposome drug delivery system, whereas IGF-I in the free form was cleared in one day (Katre et al., 1998).

Sustained delivery systems based on the biodegradable polymers poly(lactic acid) (PLA) and poly(lactic/glycolic acid) (PLGA) have also been demonstrated to be effective in several *in vivo* studies. Heya et al. demonstrated the potential of PLGA microspheres as a sustained delivery system for thyrotropin releasing hormone (TRH) (Heya et al., 1994) and Okada et al. developed and evaluated a PLGA microsphere sustained delivery system for leuprorelin (Okada, 1997; Okada et al., 1994). In addition, long-acting delivery systems for triptorelin and other LHRH agonists in microcapsules of poly(DL-lactide-co-glycolide) (PLG) or different polymers designed to release a controlled dose of the peptide over a 30-day period were developed (Parmar et al., 1985; Sharifi et al., 1990).

Another approach to achieve sustained delivery of peptide and protein drugs is the utilization of emulsions. A water/oil emulsion containing the hydrophilic markers aprotinin as well as pertechnetate displayed enhanced *in vivo* retention (Bjerregaard et al., 2001).

Also cyclodextrins and derivates are known for several years to provide a sustained release of peptide drugs. Uekama et al. for example demonstrated a significantly retarded release of buserelin *in vitro* in presence of heptakis (2,6-di-O-ethyl)-beta-cyclodextrin (DE-β-CyD) as well as an effective continuous plasma level of buserelin *in vivo* after single subcutaneous injection of a suspension containing a buserelin-DE-β-CyD complex. Figure 3 shows the plasma concentrations of buserelin in rats following the subcutaneous administration of the oily suspension (Uekama et al., 1989).

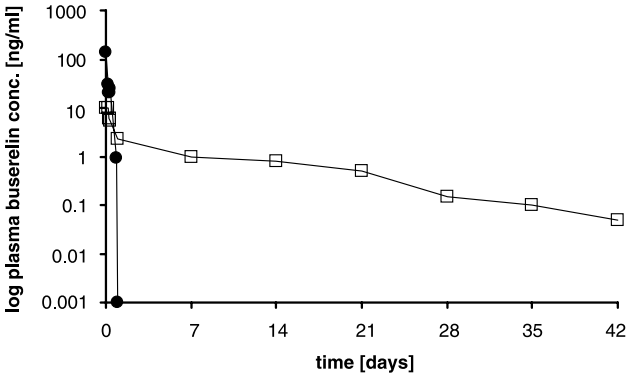

Fig. 3. Plasma levels of buserelin following the subcutaneous administration of the oily suspension containing buserelin acetate or its DE-β-CyD complex (equivalent to 1 mg/kg as buserelin acetate) to rats. ○: Buserelin acetate; ●: DE-β-CyD complex; each point represents the mean ± s.e. of 5 rats; modified from Uekama et al. (1989)

4. Conclusion

To design long-acting analogues of peptide and protein drugs as well as delivery systems, it is of great importance to know the 'enemies strength'. Taking the different strategies to prolong half-life time discussed within this review into consideration, drug modifications should be based on an exact knowledge of the influence of proteolytic enzymes encountered with systemic circulation as well as the renal clearance of the drug. Within this review, an overview of most important proteolytic enzymes of human blood, liver and kidney as well as their cleavage specificity and inhibitors is provided in order to predict theoretical cleavage of peptide and protein drugs during systemic circulation. Furthermore, methods and strategies discussed within this review should allow well directed peptide/protein drug modification to prolong plasma half life time.

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