Prodrug strategies to enhance the intestinal absorption of peptides

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Clinical development of orally active peptide drugs has been restricted by the unfavorable physicochemical properties of these molecules limiting intestinal mucosal permeation and the lack of stability of peptides against enzymatic degradation. Successful oral delivery of peptides will depend, therefore, on strategies designed to alter the physicochemical characteristics of these potential drugs, without changing their biological activity, in order to increase the permeation across intestinal cells. This manuscript will focus on the biological barrier properties that limit oral peptide bioavailability and on prodrug strategies designed to overcome these barriers.

Recent dramatic advances in molecular biology and modern synthetic chemistry have generated new methodologies that permit the production of large quantities of structurally diverse peptides possessing a wide range of pharmacological effects. The clinical development of drugs in this structural class, however, has been restricted because of unfavorable permeation characteristics across important biological barriers, including the intestinal mucosa and blood–brain barrier, and the susceptibility to enzyme-mediated degradation. These characteristics lead to oral bioavailabilities typically of less than 1–2% and short *in vivo* half-lives (<30 min)[1–6].

The successful design of peptides as orally bioavailable drugs will represent a major challenge to pharmaceutical scientists in the future. In order to develop an orally bioavailable peptide drug having high specificity and *in vivo* potency, it will be necessary to incorporate structural features that will optimize the pharmacological (e.g. receptor binding), pharmaceutical (e.g. solubility) and biopharmaceutical (e.g. membrane permeability, metabolic stability) properties of the molecule. Alternatively, unfavorable pharmaceutical and/or biopharmaceutical properties of the molecule can be transiently modified using various prodrug approaches. In this manuscript, we will focus on the biological barriers that limit oral peptide bioavailability and on prodrug strategies designed to overcome these barriers.

Biological barriers limiting oral peptide bioavailability

The major biological barriers limiting the oral delivery of peptide-based drugs include the intestinal lumen, intestinal mucosa and the liver. This review will concentrate on the role of the intestinal lumen and the intestinal mucosa as significant barriers. The role of the liver in limiting the oral bioavailability of peptide drugs has been reviewed in depth elsewhere recently[7–9].

Intestinal lumen

The physiological function of the gastrointestinal tract is to digest macromolecules, such as proteins, and transform them into smaller subunits (e.g. di-/tripeptides, amino acids) that can be absorbed easily[10]. Digestive processes for proteins are catalyzed by a variety of enzymes specialized in the

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  1359-6446/97/$17.00. PII:S1359-6446(97)01011-8

hydrolysis of peptide bonds. Because of the broad substrate specificities of these proteases and peptidases, the metabolic activity in the intestinal lumen forms a significant barrier to the absorption of peptide-like drugs.

Upon reaching the duodenum, peptide degradation can be mediated by pancreatic proteases in the lumen. The relative importance of this luminal hydrolysis in the overall degradation is dependent on the size and the primary sequence (i.e. the amino acid composition) of the peptide[11]. However, significant degradation (>30%) of a peptide is assumed to require at least contact with the brush-border membrane and/or uptake into the intestinal mucosal cells and metabolism by enzymes associated with these epithelial cells.

Intestinal epithelial cells

The epithelium lining of the gastrointestinal tract acts as a strategic interface between the external environment and the internal milieu of the body. The barrier properties of the intestinal mucosa consist of both physical and biochemical components.

The physical barrier properties arise from the tight intercellular junctions and the lipid matrix of the membrane. The organization and architecture of the intestinal mucosa restrict peptide permeation across this cell barrier to the paracellular and/or the transcellular routes (Figure 1). The paracellular pathway is an aqueous extracellular route across the epithelium that has gained substantial attention for the delivery of peptide drugs because of the perception that it is deficient in proteolytic activity[12,13]. Examples of biologically active peptides that are assumed to permeate the intestinal mucosa predominantly via this pathway include octreotide[14,15], thyrotropin-releasing hormone (TRH)[16], and 1-deamino-8-D-arginine vasopressin (DDAVP)[17], a potent analog of vasopressin. Translocation of solutes via the paracellular route takes place primarily by passive diffusion according to a gradient originated from differences in concentration, electrical potential and hydrostatic pressure between the two sides of the epithelium[18].

The main barrier to the paracellular diffusion of molecules is the region of tight junctions or zonula occludens, which is discussed in detail elsewhere[18–20]. In a recent study, our laboratory investigated the effect of size and charge of metabolically stable hydrophilic peptides across the intestinal mucosa using the Caco-2 cell culture model[21]. Apparent permeability coefficients calculated for charged model peptides increased on average by a factor of two when the molecular

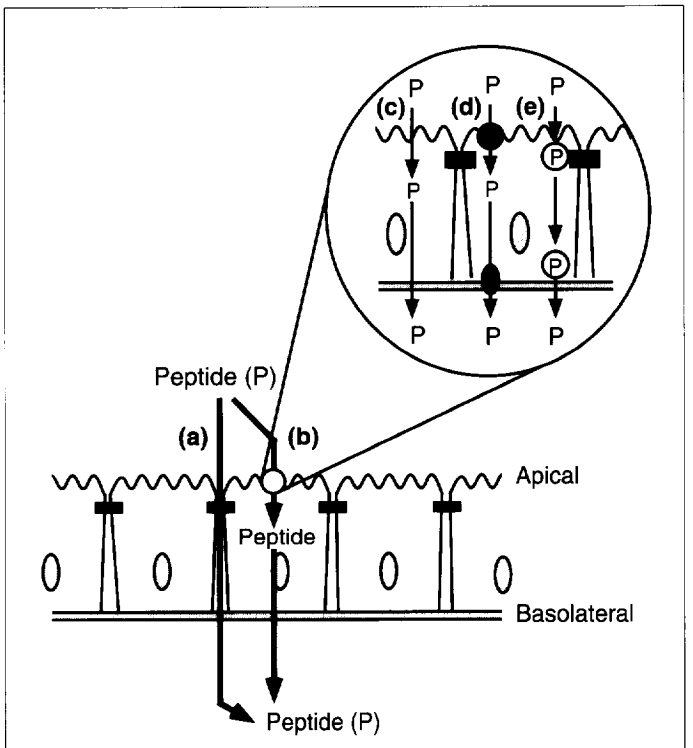

Figure 1. *Physical barrier properties of the intestinal mucosa limit peptide flux to (a) paracellular and (b) transcellular pathways. The transcellular pathways for intestinal absorption of peptides include (c) passive diffusion (d), carrier-mediated absorption, and (e) vesicular transport mechanisms.*

size was reduced from a hexapeptide to a tripeptide and an amino acid, respectively. Because of ionizable side chains in the tight junction proteins, the junctional space exhibits an electrostatic field with a negative net charge that may affect the paracellular flux of molecules via charge–charge interactions. For small hydrophilic peptides (≤ tripeptides), transmucosal transport is more favorable for positively charged peptides than it is for negatively charged molecules[21]. At the level of hexapeptides, however, the charge selectivity of the cell monolayer (i.e. positive > negative) was almost negligible, which suggests that the size sieving by the pores becomes more dominant with increasing molecular size.

The transcellular pathway involves movement of the solute across the apical cell membrane, through the cytoplasm and across the basolateral membrane by active and/or passive processes (Figure 1). In general with peptides, transcellular flux by passive diffusion is minimal[22] because of the predominantly hydrophilic nature of biologically active peptides. However, increasing the lipophilicity of the molecule can make this pathway more attractive for

delivery. Recently, our laboratory has shown that cyclic analogs and cyclic prodrugs of peptides were more permeable in Caco-2 cell monolayers, an *in vitro* model of the intestinal mucosa, than were the corresponding linear peptides[23,24]. It was found that because of cyclization, peptides could form intramolecular hydrogen bonds, which increased their lipophilicities[25,26].

Traditionally, lipophilicity has been viewed as the most important molecular characteristic in determining passive diffusion through biological membranes. Nevertheless, early *in vivo* data suggest that intestinal absorption may decline when lipophilicity becomes too high[27]. These results imply an 'optimal' rather than a high lipophilicity for improved transmucosal permeation of a molecule. It has been demonstrated for a variety of small organic molecules that the octanol/water partition coefficient ($P_{o/w}$) is a good predictor of the permeation across a biological membrane[28–30]. With peptide drugs, however, Conradi and coworkers[31] have shown that the lipid solubility as expressed by the $P_{o/w}$ did not correlate with membrane permeation characteristics. A better predictor was the hydrogen bonding potential of the peptides, which was experimentally assessed by the difference in partition coefficients determined in the octanol/water and the isooctane/water systems. The same conclusion was drawn when the transport of the model peptides was studied in an *in situ* perfused rat ileum model[32].

Active processes (e.g. via the di-/tripeptide transporters) are fairly substrate-specific and, therefore, difficult to target for the transport of non-substrates. Nevertheless, compounds that are transported via these oligopeptide transporters (for example cephalosporins) have been reported[33]. As a consequence, research has focused on the identification of structural requirements necessary for binding and transport mediated by these transporters, with the objective of using oligopeptide transporters for the delivery of drugs. Examples of successful targeting of the oligopeptide transporters to enhance the transport of molecules across the intestinal mucosa include a pGlu-L-dopa prodrug[34], angiotensin-converting enzyme (ACE) inhibitors[35] and thrombin inhibitors[36]. Structural features that influence the carrier-mediated transport of these peptides have been reviewed elsewhere recently[6,37]. Briefly, the C-terminal carboxylic acid of a peptide appears to be important for recognition as a substrate for oligopeptide transporters. Modification of the C-terminal carboxyl group generally leads to reduction or total loss of the affinity to the transporters[38,39]. The effect of the net charge on the affinity of a dipeptide for the intestinal oligopeptide transporters is still controversial[39–41]. With respect to stereoselectivity, it appears that only one L-amino acid must be present in di- or tripeptides in order to show affinity for the intestinal oligopeptide transporters. Nevertheless, transport studies across Caco-2 cell monolayers performed with stereoisomers of Val-Val and Val-Val-Val (Refs 42,43) revealed that the major transepithelial transport mechanism for these isomers is passive diffusion via the paracellular route, and that the carrier-mediated transport via oligopeptide transporters represents only a minor fraction of the overall peptide flux.

Cellular internalization of polypeptides by endocytosis is another important biological transport process. Peptides too large to be absorbed via the di-/tripeptide transport systems can be taken up into the intestinal cells by either nonspecific fluid-phase endocytosis (pinocytosis) or receptor-mediated endocytosis, which requires binding of the peptide to the plasma membrane before it is incorporated into endocyte vesicles. Finally, polypeptides can be carried in endosomes directly to the basolateral side (i.e. bypassing the lysosomes), where they are released into the extracellular space. This process is known as transcytosis. Although there is some evidence that mucosal peptide/protein uptake is mediated by endocytic processes[44,45], in most instances this does not lead to transcytosis. In contrast, a specialized type of undifferentiated crypt cell in the Peyer's patches, called M-cell, has significant ability to transcytose polypeptides. Its function and potential role in oral polypeptide absorption have been described recently[13].

Although the physical barrier of the intestinal mucosa can be attributed predominantly to the cell layer, additional factors may hinder the passage of peptides. The intestinal mucosa is coated, like most epithelial surfaces, with a layer of mucus, which serves as a lubricant and protective barrier. Mucus, in reality, is a constantly changing mix of many secretions, including exfoliated epithelial cells[46]. The main determinants of the physical and functional properties of mucus secretions are high-molecular-weight glycoproteins, termed mucins. Much research has been done to understand the regulatory mechanisms of mucin secretion and its role in the modulation of tissue function[47]. However, the role of the mucus layer as a physical barrier in the absorption of peptides from the gastrointestinal tract is not well established.

Biochemical barrier

Because pancreatic proteases in the lumen of the intestinal tract are active mainly against dietary proteins and not

toward small peptides[12], significant enzymatic degradation of peptide drugs appears to be mediated by proteases associated with the enterocytes, the epithelial cells that form the intestinal lining (Figure 2). Although lysosomes and other organelles may act as potential sites of peptide metabolism, proteases in the brush-border membrane are probably the biggest deterrent to the absorption of small peptides across the intestinal mucosa[11,48]. Brush-border peptidases and proteases are active predominantly against tri-, tetra- and higher peptides with up to ten amino acid residues[49,50], while intracellular enzymes mainly attack dipeptides[49–51]. Many intestinal peptide hydrolases are characterized and listed under the formal enzyme classification (EC) system based on their site of action in a susceptible substrate [e.g. aminopeptidase N (EC 3.4.11.2), carboxypeptidase P (EC 3.4.17) and dipeptidyl peptidase IV (EC 3.4.14.5)]. For a more detailed compilation of intestinal peptidases with their individual substrate specificities, the interested reader is referred to the publications by Barrett and McDonald[52,53] as well as by Bond and Beynon[54].

The regional distribution of the brush-border exopeptidases, aminopeptidase P (EC 3.4.11.9) and aminopeptidase W (EC 3.4.11.16) has been studied in rat and rabbit[55]. The activity of these enzymes increases distally and

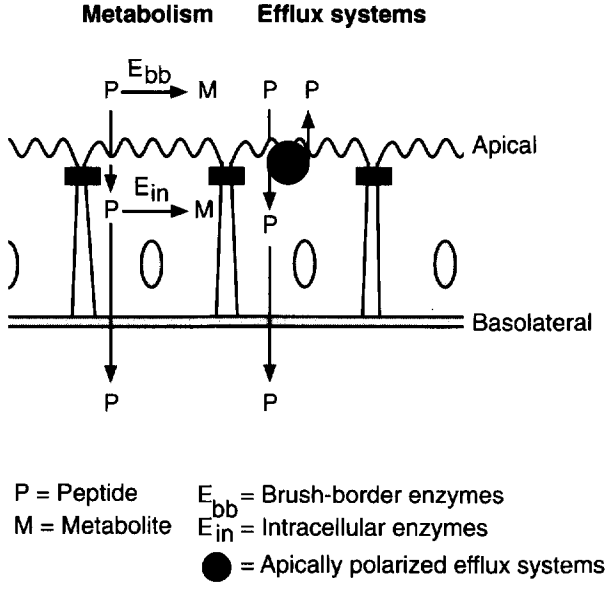

Figure 2. *The biochemical barrier reduces transport of peptides through the intestinal mucosa by brush-border and/or intracellular metabolism and by apically polarized efflux systems.*

reaches its highest level in the ileum. The lowest activities, however, were measured in the ileocecal junction. Similar results were found in rabbits as well as in human for aminopeptidase N (EC 3.4.11.2) and dipeptidyl peptidase IV (EC 3.4.14.5)[56,57]. In contrast, the activities of cytosolic peptidases do not show any regional variation[58]. Lysosomal peptidases, on the other hand, seem to have their highest activities predominantly in the cecum[59].

Peptidase activity in the gastrointestinal tract is reported to be sensitive to various factors including age[60], diet[61,62] and administration of drugs[63]. However, the impact of these factors on the absorption of biologically active peptides has not been extensively explored.

Efflux systems

In recent years, it has been found that the barrier function of the intestinal mucosa cannot be adequately described by a combination of the metabolic and physical barriers alone. Efflux systems (Figure 2), which are known to be present in cancer cells and represent a major barrier to the uptake of a wide variety of chemotherapeutic agents (i.e. in multidrug resistance), have also been identified in normal intestinal and colonic cells[64]. Some of these efflux systems seem to be related to P-glycoprotein, the principal component of multidrug resistance in a variety of cell types. P-glycoprotein is a 170–180 kDa membrane glycoprotein acting as an ATP-dependent efflux pump that reduces the intracellular accumulation and/or the transcellular flux of a wide variety of drugs, including peptides (e.g. gramicidin D, valinomycin)[65]. Because of the polarized expression of these efflux systems, it is assumed that their physiological role is to restrict transcellular flux of some molecules. Therefore, they serve as a major barrier in the gastrointestinal epithelium by limiting the absorption of drugs, including peptides[66,67].

Prodrug strategies to improve oral peptide bioavailability

As described above, the clinical development of orally active peptide drugs has been limited by unfavorable physicochemical characteristics, such as size, charge and hydrogen bonding potential, that prevent peptides from permeating biological barriers, such as the intestinal mucosa, and the lack of stability against enzymatic degradation. Unfortunately, many of the structural features of peptides, such as the N-terminal amino group and C-terminal carboxyl group, and side chain carboxyl, amino and hydroxyl

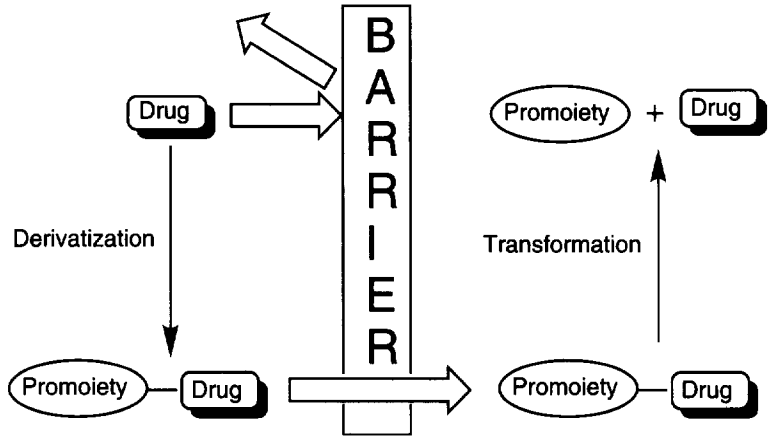

Figure 3. *The prodrug concept.*

groups, that give the molecule its affinity and specificity for the pharmacological receptor, severely restrict its ability to permeate biological barriers and make it a substrate for peptidases. For small organic drugs, which exhibit similar structural functionalities and similar unfavorable physicochemical characteristics, prodrug strategies have been successfully employed to transiently alter the physicochemical characteristics of the drugs as well as the lability to metabolism[68–74] Prodrugs are molecules that must undergo chemical or biochemical conversion to the active drug before exerting pharmacological effects. The major goal in designing prodrugs is to overcome some of the limitations of the parent drug, including poor solubility, poor chemical and/or enzymatic stability, poor membrane permeability, rapid elimination by the liver or kidney, and lack of targeted delivery. The prodrug concept is illustrated in Figure 3. Unfortunately, the synthesis of peptide prodrugs has been limited because of their structural complexity and the lack of novel methodology[75].

The prodrug approach has been shown to work quite nicely in the case of an orally active platelet fibrinogen receptor (GP IIb/IIIa) antagonist (Figure 4)[76]. The amidoxime ester prodrug of a nonpeptide GP IIb/IIIa antagonist (Ro48-3657) was shown to be absorbed approximately 20 times better after oral administration to mice than was the parent drug. Kim and coworkers have prepared the ester prodrug of an ACE inhibitor, benazepril (Figure 4), and have shown that the uptake rate of the more lipophilic prodrug was twice as great as that of the parent drug[77]. Bundgaard and his colleagues have prepared several pivalate ester prodrugs of desmopressin (Figure 4), a synthetic analog of the antidiuretic hormone vasopressin[78]. These derivatives were shown to convert quantitatively to desmopressin by enzymatic hydrolysis in human plasma. The transport of the pivalate prodrug across monolayers of Caco-2 cells was found to be remarkably higher than that of desmopressin. Bundgaard and coworkers have also synthesized other prodrugs of peptides, mainly by introducing chemical modifications at the side chain or the peptide termini (e.g. *N*-alkoxycarbonyl derivatives at the imidazole group of the histidine residue on TRH)[79]. These prodrugs have been shown to be more lipophilic than the parent peptides and significantly more

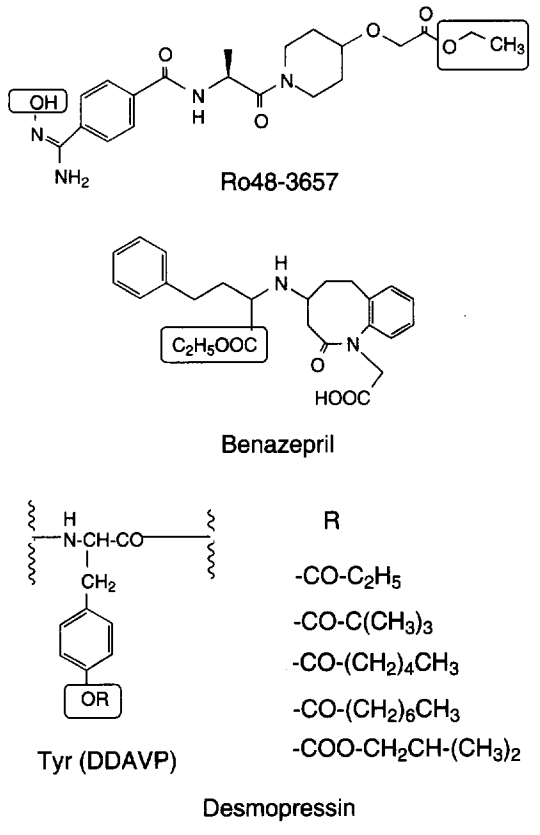

Figure 4. *Recent examples of prodrugs of peptides and peptidomimetics that resulted in a significant increase in permeation across the intestinal mucosa when compared with the parent drug. The functional groups added to the parent drugs to create the prodrugs are shown in boxes (DDAVP, 1-deamino-8-D-arginine vasopressin).*

stable toward enzymatic degradation. Unfortunately, no transport data are available for these peptide prodrugs across the intestinal epithelium either *in vitro* or *in vivo*.

Bioreversible cyclization

Bioreversible cyclization of the peptide backbone is one of the most promising and intriguing new approaches to the development of peptide prodrugs. Cyclization of the peptide backbone enhances the extent of intramolecular hydrogen bonding and reduces the potential for intermolecular hydrogen bonding to aqueous solvents. Conradi and coworkers have shown that a reduced number of potential hydrogen bonding sites in peptides ultimately increases the permeation characteristics of these molecules across Caco-2 cell monolayers[31]. In addition, cyclization of the backbone blocks the C-terminal carboxyl group and N-terminal amino group and protects the peptide from amino- and carboxypeptidases.

Our laboratory has taken advantage of this peptide backbone cyclization strategy and prepared cyclic esterase-sensitive prodrugs of a model peptide, H-Trp-Ala-Gly-Gly-Asp-Ala-OH, using two different promoieties[23,25,80–82]. These prodrug systems were prepared by linking the N-terminal amino group to the C-terminal carboxyl group via an acyloxyalkoxy promoiety (**1**)[83,84] or a 3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid promoiety (**2**) (Figure 5). These cyclic prodrugs were designed to be susceptible to esterase metabolism (slow step) leading to a cascade of chemical reactions and resulting in generation of the linear peptide (Figure 5).

In pH 7.4 buffer [Hank's balanced salt solution (HBSS)] at 37 °C, both cyclic prodrugs were shown to degrade quantitatively to the hexapeptide[23,81]. The rate of hydrolysis of cyclic prodrugs was significantly faster in human blood than in HBSS. In human blood, cyclic prodrugs were shown to be substantially more stable than the linear hexapeptide. Transport studies were conducted using monolayers of Caco-2 cells[23,81]. In comparison with the linear hexapeptide, both cyclic prodrugs were found to be at least 70 times more able to permeate the cellular barrier of this cell culture model of the intestinal mucosa.

In addition, we have been able to correlate enhanced cellular permeability to the structural features of cyclic prodrug **1** by using NMR, CD and molecular dynamics simulation studies[25]. These studies indicate that the cyclic prodrug **1** exhibits one major conformer in solution and has a well-defined secondary structure. The solution conformation of cyclic prodrug **1** appears to be compact as a result of intramolecular hydrogen bonding and the presence of a β-turn (Figure 6). The increased ability of cyclic prodrug **1** to permeate membranes could be due to reductions in the average hydrodynamic radius of the molecule, thus increasing paracellular flux, and/or to an increase in passive diffusion via the transcellular

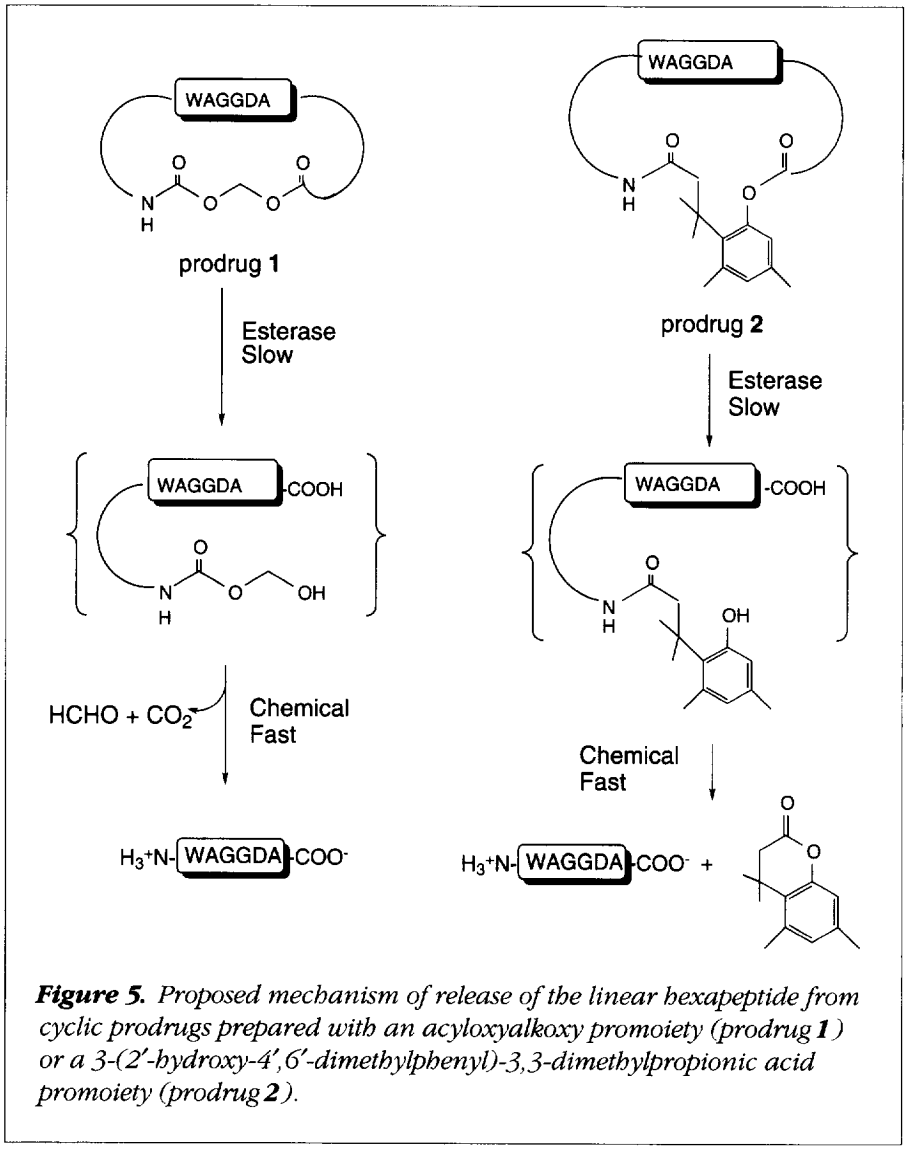

Figure 5. *Proposed mechanism of release of the linear hexapeptide from cyclic prodrugs prepared with an acyloxyalkoxy promoiety (prodrug **1**) or a 3-(2′-hydroxy-4′,6′-dimethylphenyl)-3,3-dimethylpropionic acid promoiety (prodrug **2**).*

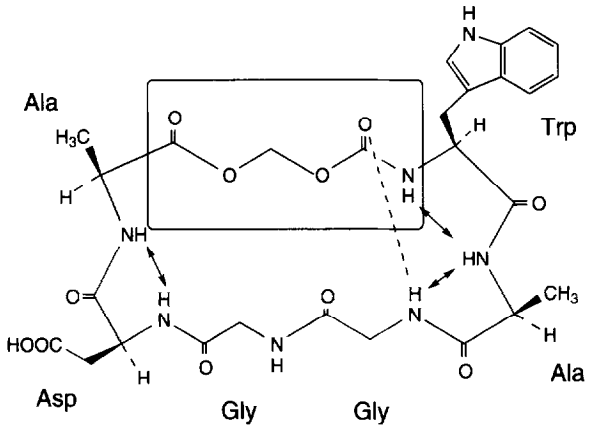

Figure 6. *Secondary structure of cyclic prodrug **1** in solution (from Ref. 25, with permission). The dashed line indicates the intramolecular hydrogen bond of a β-turn and arrows symbolize the close proximity (≤4 Å) of amide protons in the peptide backbone.*

route because of a reduction in the hydrogen bonding potential of the cyclic prodrug.

Currently in our laboratory, we are using this methodology to cyclize other biologically active peptides by linking the C-terminal carboxyl group to a side chain amino (e.g. Lys, Arg) or hydroxyl (e.g. Ser, Thr, Tyr) group, or by linking a side chain carboxyl group (e.g. Asp, Glu) to a side chain amino (e.g. Lys, Arg) or hydroxyl (e.g. Ser, Thr, Tyr) group.

In this brief review, we have summarized the applicability of the prodrug concept to improve oral delivery of peptide and peptide-based drugs. Recent examples clearly demonstrate that oral bioavailability of peptide drugs can be significantly improved by using various prodrug approaches that lead to increased metabolic stability and enhanced membrane permeation characteristics of these drugs. However, prodrug research needs more imagination and less dependency on what has been tried in the past. We hope that this article will energize renewed interest in prodrug strategies to improve the oral delivery of peptide-based drugs.

ACKNOWLEDGEMENTS

The authors' research in this area has been supported by research grants from the United States Public Health Services (DA-09315, GM-51633, GM-088359), Glaxo Wellcome, Inc., Costar Corporation and the Swiss National Science Foundation.

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