Peroral Route: An Opportunity for Protein and Peptide Drug Delivery†

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I. Introduction

The better understanding of endogenous proteins, peptides, and peptidergic molecules and their role in various body functions and pathological conditions in last few decades has resulted in realization of the enormous therapeutic potential of proteins and peptides (PPs). As a consequence, a variety of new PP drugs have been developed which offer the advantages of being very potent and specific therapeutic agents.[1] Initially, use of PPs as pharmaceuticals was severely limited, as they were difficult to produce and were isolated from animal sources. These PP products obtained from animals differed from functional molecules present in the human body, and their use as therapeutic agents raised concerns with regard to their immunogenic potential.[2,3] As a result of intensive research efforts in both academic and industrial laboratories, recombinant DNA, protein engineering, and tissue culture techniques can now be used to obtain PPs, on a commercial scale, which resemble endogenous molecules and thus provoke fewer or minimal immunological responses. Additionally, due to advances in analytical separation technology, recombinant proteins can now be purified to unprecedented levels.[4] Today, PPs along with informational macromolecules normally produced by the body including endorphins, enkephalins, leutinizing hormone releasing hormone, and interferons form an increasingly important class of therapeutic agents. Table 1 lists PP products introduced in the market over the past few years.[5−8]

Though the initial problems related to obtaining nonimmunogenic PP drugs in purer form at commercial scales have been overcome to quite some extent,[9] their formulation and optimum delivery still remain as the biggest challenges to pharmaceutical scientists. Use of PPs as therapeutic agents is limited due to lack of an effective route and method of delivery. Various critical issues associated with PP delivery that have drawn the attention of formulation scientists include the following. (i) PPs are high molecular weight biopolymers which serve as enzymes, structural elements, hormones, or immunoglobulins and are involved in several biological activities. However, due to their large molecular weight and size, they show poor permeability characteristics through various mucosal surfaces and biological membranes.[10−12] (ii) Many PP drugs are efficacious, in large part because of their tertiary structure. The tertiary structure can be lost under various physical and chemical environments, resulting in their denaturation or degradation with consequent loss in biological activity, hence, making these molecules inherently unstable.[8,13,14] (iii) Many PPs have very short biological half-lives in vivo due to their rapid clearance in liver and other body tissues by proteolytic enzymes.[15−17] (iv) As PP drugs have very specific actions and are highly potent, precise clinical dosing is of utmost importance.[18]

The most important consideration when designing an effective delivery system for any drug is that of achieving a predictable and reproducible absorption into systemic circulation with high bioavailability. In the case of PP drugs, an interplay of poor permeability characteristics, luminal, brush border, and cytosolic metabolism, and hepatic clearance mechanisms results in their poor bioavailability from oral

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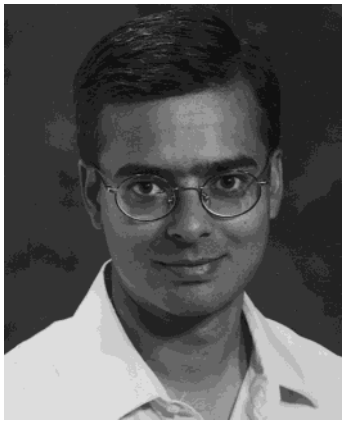
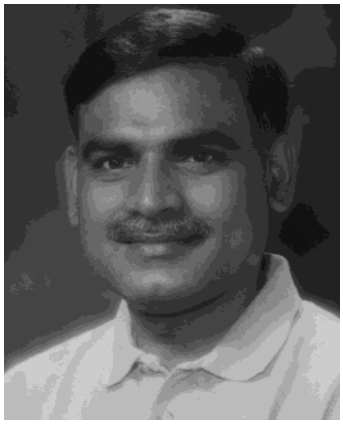


Anurag Sood was born in 1972. He received his B.Pharm and M.Pharm degrees from Dr. Hari Singh Gour Vishwavidyalaya, Sagar, India. After finishing his Master's degree, he joined Dr. Reddy's Research Foundation, Hyderabad, India, as Trainee Pharmacologist, where he was a part of team responsible for in vivo characterization of potential drug candidates for pharmacokinetics and metabolism. Presently, he is a research scholar at National Institute of Pharmaceutical Education and Research (NIPER), India. He has been a recipient of a Junior Research Fellowship (1994–96) from the University Grants Commission (UGC), India, and a Senior Research Fellowship (1998 onward) from the Council of Scientific and Industrial Research (CSIR), India. His research activities include design, development, and evaluation of conventional and novel *peroral*-controlled release drug delivery systems. Various drug delivery systems, drug release kinetics and mechanisms, and analytical method development and validation are the topics of his interest. He has 13 publications to his credit and has presented his research at various national and international conferences. He is a recipient of the G. P. Nair Award of the Indian Drugs Manufacturer's Association (IDMA) for securing first rank in his B.Pharm degree course at University. He is fond of music and loves listening to Hindi and Urdu Ghazals during his leisure time.



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and nonoral mucosal routes.[19] Hence, at present these drugs are usually administered by parenteral route. However, inherent short half-lives of PPs and almost warranted chronic therapy requirements in a majority of cases make their repetitive dosing necessary. Frequent injections, oscillating blood drug concentrations, and low patient acceptability make even the simple parenteral administration of these drugs problematic. This has prompted researchers to develop new delivery systems which can effectively deliver this important class of drugs.[20−30] Although there have been reports of successful delivery of various PP therapeutics across non-*peroral* mucosal routes,[31,32] *peroral* route continues to be the most intensively investigated route for PP administration. This interest in the *peroral* route, despite enormous barriers to drug delivery that exist in the gastrointestinal tract (GIT), can be very well appreciated from obvious advantages such as ease of administration, large patient acceptability, etc. Potential cost savings to the health care industry further augment the advantages of *peroral* systems in terms of patient compliance and acceptability, since *peroral* formulations do not require sophisticated sterile manufacturing facilities or the direct involvement of health care professionals. There have been efforts to circumvent the gastrointestinal (GI) absorption barriers to PP drugs since the 1920s, when insulin was used first as a therapeutic protein, however only with a limited success.[33−38] After the success of *peroral* cyclosporin formulations,[39−41] the efforts in this field have further intensified. There are a plethora of attempts and reports wherein the use of different approaches for *peroral* PP delivery has been investigated. The purpose of the present review is to examine recent developments in *peroral* PP drug delivery. Various barriers to PP drug absorption have been discussed in brief with attention particularly focused on drug delivery approaches that have been used or are being developed to overcome these barriers. The reports of successful improvement of *peroral* bioavailability of PPs and mechanisms involved therein are emphasized the most.

II. Barriers to Peroral Delivery of PP Drugs

The *peroral* route poses significant challenges for PP drug delivery. The barriers to PP absorption from GIT are primarily chemical, enzymatic, as well as penetration related. Acid-induced hydrolysis in the stomach, enzymatic degradation throughout the GIT by several proteolytic enzymes, bacterial fermentation in the colon, and physical barriers to absorption are traditionally believed to prevent the *peroral* delivery of PPs (Table 2). However, the nature of these barriers has now been expanded to include intracellular metabolism by cytochrome P450−3A4 as well as apically polarized efflux mediated by ATP-dependent P-glycoproteins.[42−44] Although, P-glycoprotein-mediated efflux systems are most commonly observed in tumor cells, they are also present in normal intestinal cells and act to reduce the intra-

Table 1. PP Drug Products Approved in the United States over the Last Few Years

product name	protein/peptide	company
Actimmune	Interferon gamma-1b	InterMune Pharmaceuticals
Activase	Alteplase recombinant	Genentech
Adagen	Pegademase bovine	Enzon
Alferone N	Interferon alfa-n3	Interferon Sciences
Avonex	Interferon beta-1a	Biogen
BeneFIX	Recombinant human factor IX	Genetics Institute
Betaserone	Interferon beta	Chiron/Berlex
BioTropin	Human growth hormone	Bio-Technology General
Bioclate	Recombinant antihemophilic factor	Centeon
CEA-Scan	Technetium-99m-arcitumomab	Immunomedics
Cerezyme	Recombinant glucocerebrosidase	Genzyme
Comvax	Recombinant vaccine	Merck
Crofab	Crotalidae polyvalent immune Fab (ovine)	Protherics
Enbrel	Recombinant soluble receptor	Immunex
Engerix-B	Hepatitis B vaccine recombinant	SmithKline Beecham
EPOGEN	Epoetin alfa	Amgen
Follistim	Recombinant follicle-stimulating hormone	Organon
GenoTropin	Somatropin	Pharmacia & Upjohn
Geref	Human growth hormone releasing factor	Serono Laboratories
Gkucagen	Recombinant glucagons	Novo Nordisk
Gonal-F	Recombinant human follicle stimulating hormone	Serono Laboratories
Helixate	Recombinant antihemophilic factor	Centeon
Herceptin	Anti-breast cancer MAb[3]	Genentech
Humalog	Insulin lispro	Eli Lilly
Humate-P	Antihemophilic factor	Centeon
Humatrope	Somatropin	Eli Lilly
Humulin	Human insulin (recombinant DNA origin)	Eli Lilly
Infergen	Interferon alfacon-1	Amgen
Intron	Interferon alfa-2b	Schering-Plough
KoGENate	Recombinant anti hemophilic factor	Bayer Corporation
Leukine	GM-colony stimulating factor	Immunex
LYMErix	Recombinant OspA	SmithKline Beecham
MYOBLOC	Botulinum toxin type B	Elan
MyoScint	Imiciromab pentetate, Mab	Centocor
Nabi-HB	Hepatitis B immune globulin (human)	Nabi
Neumega	Oprelvekin, Mab	Genetics Institute
NEUPOGEN	Filgrastim	Amgen
Norditropin	Somatropin	Novo Nordisk
Novolin	Recombinant insulin	Novo Nordisk
Nutropin AQ	Somatropin	Genetech
Nutropin Depot		
Nutropin	Somatropin	Genentech
OncoScint	Satumomab pendetide, Mab	Cytogen
Oncospar	PEG-L-asparaginase	Enzon
Ontak	Denileukin diftitox	Ligand Pharmaceuticals
Orthoclone OKT 3	Muromonab-CD3, Mab	Ortho Biotech
PEG-Intron	Peginterferon alfa-2b	Schering Corporation
Prevnar	Diphtheria CRM197 Protein	Lederle
Procrit	Epoetin alfa	Ortho Biotech
Proleukin	Interleukin-2	Chiron
ProstaScint	Capromab pentitate, Mab	Cytogen
Protropin	Somatrem	Genentech
Pulmozyme	Recombinant dornase alfa	Genentech
Rebetron	Ribavirin/interferon alfa-2b combination	Schering-Plough
Recombinate	Recombinant anti hemophilic factor	Baxter Healthcare
RECOMBIVAX HB	Recombinant hepatitis B vaccine	Merck
ReFacto	Recombinant antihemophilic factor	Genetics Institute
Refludan	Lepuridin	Aventis
Regranex	Becaplermin	Ortho-McNeil
Remicade	Infliximab, Mab	Centocor
ReoPro	Abciximab, anti-platelet Mab	Centocor/Eli Lilly
Retavase	Reteplase	Centocor
Rituxan	Ritiximab, Mab	Genentech
Roferone−A	Recombinant interferon alfa-2a	Hoffmann-La Roche
Saizen	Somatropin	Serono laboratories
Serostim	Somatropin	Serono Laboratories
Simulect	Basiliximab, Mab	Novartis
Synagis	Palivizumab, Mab	MedImmune
Thymoglobulin	Thymocyte globulin, polyclonal antibody	SangStat
Thyrogen	Thyrotropin alfa	Genzyme
TNKase	Tenecteplase	Genentech
Verluma	Nofetumomab, MAB	DuPont Merck
Wellferone	Interferon alfa-n1	Glaxo Wellcome
Zenapax	Daclizumab, Mab	Hoffman-La Roche

Table 2. Various *Peroral* Absorption Barriers and Their Bearing on PP Drug Absorption from GIT

barrier nature	location and description	effect on PP drug absorption
chemical	acidic environment in stomach (pH 1.2−3.0) and alkaline environment in intestine (pH 6.5−8.0)	pH-induced oxidation, deamidation, or hydrolysis
enzymatic	luminally secreted, membrane-bound, and cytosolic proteolytic enzymes throughout the length of GI tract	proteolytic degradation in lumen and during absorption through enterocytes
	microbial flora present in colon	breakdown PP as part of their metabolic activity
physical	unstirred aqueous boundary layer and viscous mucus layer covering the surface of GI epithelial cell lining	decreased diffusion to reach absorptive epithelial cell membrane
	lipid bilayer of epithelial cell membrane	inhibits absorption of PP drugs that are hydrophilic and charged through the cell (transcellular transport)
	intercellular spaces (mean pore radii of 0.8, 0.3, and 0.3 nm in duodenum, ileum, and colon, respectively) gated by closely fitting tight junctions (TJ) on apical side of epithelial cells	TJ prevent passage of PP macromolecules through the intercellular spaces (paracellular transport)
	p-glycoprotein present on epithelial cell membrane	promote apically polarized efflux to remove permeated drug molecules

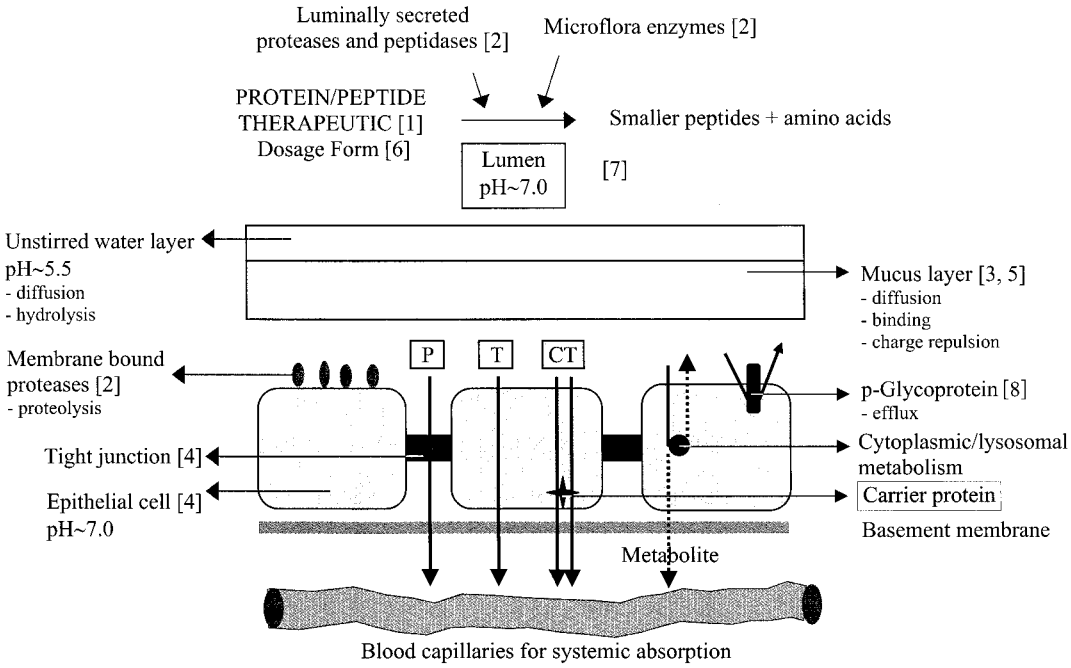


Figure 1. Diagrammatic representation of different barriers to protein and peptide drug absorption from the intestinal tract. Shaded square text boxes show the pathways for drug absorption: P, paracellular; T, transcellular; CT, carrier-mediated transport. Target sites for different absorption enhanceement strategies are indicated by numerals in paranthesis: 1, prodrugs/analogues; 2, protease inhibitors; 3, mucolytic agents; 4, paracellular and transcellular absorption enhancers; 5, mucoadesive polymers; 6, dosage form modifications; 7, pH modulation to enzymatic activity minima 8, p-glycoprotein inhibitors.

cellular accumulation or the transcellular flux of a wide variety of drugs, including peptides.[45,46] Figure 1 shows an overall view of the various barriers to PP drug absorption from *peroral* route and various targets for enhancing their absorption. A brief description of these barriers has been provided individually at appropriate places in the subsequent sections.

Traditional drug candidates also encounter similar barriers, but PP drugs seem to be highly susceptible to all these factors, and the options available to pharmaceutical scientists are very limited. The synthetic chemistry approaches that are often successful in ameliorating one or more of the barriers and resulting in efficacious in vivo absorption of traditional, small organic molecules have proved to be of little value in the case of PPs due to their much more complex chemistry. Various approaches that have been taken to overcome barriers with reference to poor bioavailability of PP drugs from *peroral* route are enumerated as follows and have been described later in the review: (i) Chemical modification of the protein or peptide lead compound—prodrug/analogue approach; (ii) Use of absorption enhancers such as surfactants, bile salts, or calcium chelators; (iii) Use of enzyme inhibitors to lower the proteolytic activity; (iv) Designing a drug delivery system which is targeted to a part of the gut where proteolytic activity is relatively low so as to protect PPs from luminal proteolytic degradation and release the drug at the most favorable site for absorption.

A. Prodrug/Analogue Approach

Prodrug or analogue development has probably remained one of the most favored approaches in solving many drug delivery related problems. The most recent example of insulin LysPro, although for parenteral administration, has demonstrated the

possibility of modifying biopharmaceutic as well as pharmacokinetic characteristics of PP drugs by using a prodrug/analogue approach. LysPro, a human insulin analogue produced by inverting the native sequence Pro^{B28}, Lys^{B29} in the c-terminal of the B-chain of human insulin,[47] was developed by Eli Lilly and Company and approved for clinical use in 1996. The sequence inversion results in reduced self-association properties of LysPro, making it more readily monomeric,[48] and consequently LysPro exhibits different pharmacokinetic properties from soluble insulin on subcutaneous administration (rapid onset, higher and earlier peak plasma concentrations with shorter duration of action).[49,50] There are a number of other insulin analogues that are presently under different phases of investigations for increasing its stability and/or modifying its onset and duration of activity.[51,52] In context to the scope of present review, the prodrug/analogue approach can be defined as conversion of PPs into derivatives (prodrugs or analogues) by means of incorporation of sufficient modifications so as to engender oral activity.[53−58] Hydrophilic nature and charge of PP drugs are because of the polar and ionizable functional groups (including terminal amino and carboxyl groups) in the molecules. The presence of amide bonds at different positions, free N-terminal amino groups, and free C-terminal carboxyl groups make them susceptible to endopeptidases-, aminopeptidases-, and carboxypeptidases-mediated degradation, respectively. Thus, chemical modification, such as masking or blocking polar amide bonds and terminal amino and carboxyl groups, primarily brings about an alteration in the physicochemical properties of drugs such as lipophilicity, hydrogen-bonding capacity, charge, molecular size, solubility, configuration, isoelectric point, chemical stability, etc., which are known to affect their membrane permeability, enzyme liability, and affinity to carrier systems.[59,60] Various structural features of peptides that influence their passive diffusion, carrier-mediated transport, and efflux mechanisms have been recently reviewed by Wang et al.[59] and Pauletti et al.[61] The lipophilicity of various drugs, as expressed in terms of logP (logarithm value of octanol−water partition coefficient) or logD (logarithm value of octanol−pH 7.4 buffer partition coefficient), can be correlated with cell membrane permeability.[62] The generalization is that within a homologous series, drug absorption increases as lipophilicity rises and is maintained at a plateau for a few units of logP after which there may be a steady decrease, giving a parabolic relation. However, in the case of PP drugs, logP or logD values may not always correlate well with drug permeability.[63] In a study with a series of six model peptides, prepared from D-phenylalanine and glycine, Conradi et al. observed that the permeability of peptides across Caco-2 cell monolayers was inversely related to the number of hydrogen-bonding groups in the structure as these hydrogen bonds must be broken for the solute to transfer into the interior of cell membrane.[64] They showed that although addition of amino acid with a large hydrocarbon chain (phenylalanine) to the peptidic chain resulted in increased lipophilicity of modified peptides, their permeability was affected adversely. The effect was explained to be due to introduction of very polar amide bonds, capable of forming strong hydrogen-bonding interactions with water, in the peptide chain with the addition of hydrophobic amino acid residue. In another study with a tetrapeptide, Conradi et al. showed that methylation of amide nitrogens resulted in a substantial increase in transport across the Caco-2 cell monolayer but without any significant change in the octanol−water partition coefficient, suggesting that a reduction in the overall hydrogen-bonding potential is more important than an increase in lipophilicity.[65] Similarly, Saitoh and Aungst showed that lipophilicity and charge of DMP-728 (a potent GP IIb/IIIa receptor antagonist) prodrugs did not influence intestinal permeability determined in vitro using rat jejunum in diffusion cells; instead, N-methyl-substituted analogues exhibited 2-fold greater jejunal permeability than DMP-728.[66] However, these observations were not always consistent with the hypothesis that reducing the hydrogen-bonding capacity of peptides can increase permeability and suggested that this could be because of confounding influence of secretory transport by P-glycoprotein. Additionally, there are a number of reports where an increase in lipophilicity, as indicated by partition coefficient values of PP molecules by means of chemical modification, has been shown to improve their membrane permeability.[53,67]

As explained earlier, PP molecules harbor more than one polar and ionizable group that contributes to the total charge and polarity of molecules and/or serves as a site for enzymatic attacks. A chemical modification at one site may not always be sufficient to significantly improve permeability characteristics and/or reduce liability to enzymatic degradation in vivo, especially when there are multiple enzymes involved in degradation at different sites. In such instances, various strategies have been tried which allow simultaneous masking of more than one functional group. Borchardt, Wang, Pauletti, and coworkers[59,68−75] described preparation of cyclic prodrugs which allow for simultaneous masking of an amino and a carboxyl group of peptide drug. These cyclic prodrug systems can be prepared by using acyloxyalkoxy-, phenolpropionic acid- or coumarine-based prodrug moieties (Table 3). Wang et al.[59] explained that cyclization of linear peptides by using these prodrug moieties results in significantly altered physicochemical properties (due to derivatization of carboxyl and amino groups into ester and amide, respectively), altered effective size and shape along with restricted conformational freedom of the cyclic peptide, which consequently reduces the charge on peptide and promotes intramolecular hydrogen bonding within the peptide molecule rather than intermolecular hydrogen bonding between peptide functional groups and solvent. These prodrugs have reduced susceptibility to peptidase metabolism; however, they are esterase sensitive and release the parent peptide under esterase activity. To achieve similar results, chemical modifications at two or three functional groups in the PP molecules have also been

Table 3. Various Approaches for Derivatization of Peptides and Proteins To Make Produgs/Analogues

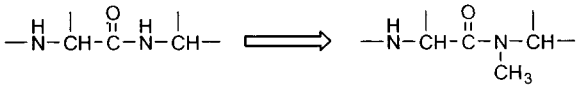
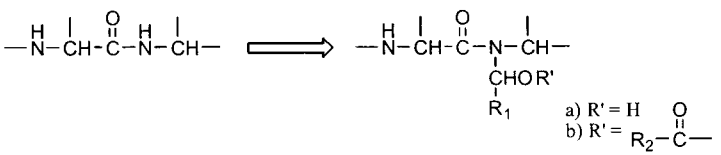
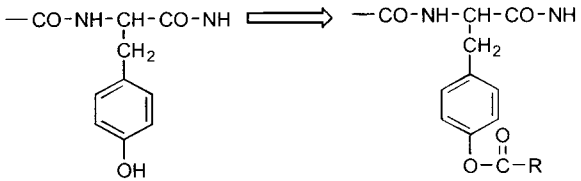
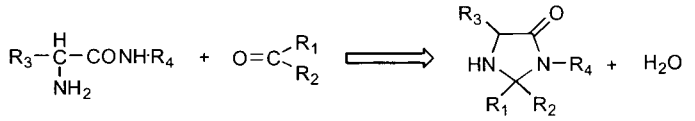
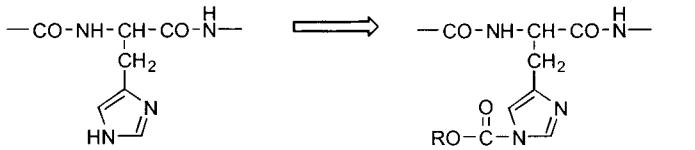
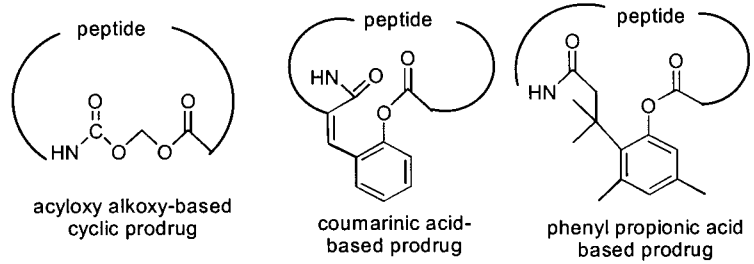
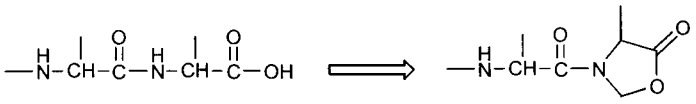
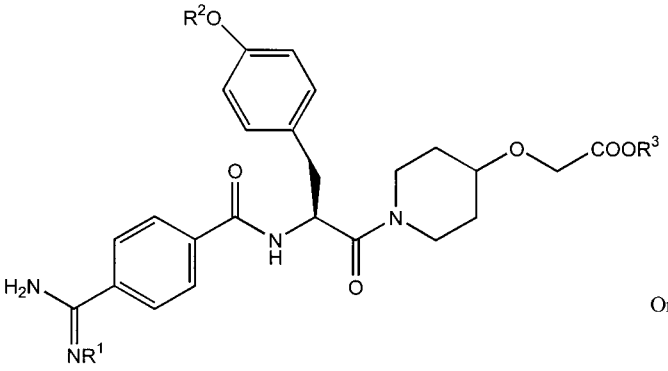
S.No.	Approach and prodrug/analogue properties	Reaction Scheme
1.	N-alkylation of peptide bonds. - Enzyme resistant. - Not bioreversible.	
2.	N-α-hydroxyalkyl (a) and N-α-acyloxyalkyl (b) derivatives peptide bond. - Resistant to carboxy peptidase and α-chymotrypsin. - Bioreversible. - N-hydroxyalkyl group on one peptide bond protects the adjoining peptide bond. - Lipophilicity is modified for (b).	
3.	Esterification of tyrosine group in tyrosine containing peptides. - Resistant to carboxypeptidases. - Bioreversible.	
4.	Peptidyl-α-hydroxyglycine derivatives with glyoxylic acid. - Resistant to a-chymotrypsin. - Bioreversible. - Besides protecting the derivatized amide moiety it can also protect the underivatized peptide bond to significant extent.	
5.	4-imidazolidinone derivatives by condensing free N-terminal group containing peptide with aldehydes or ketones. - Resistant to aminopeptidases. - Bioreversible, rate is dependent on pH and structure of R1, R2, R3 and R4. - Increased lipophilicity.	
6.	Modification of imidazole group of histidine residue, by reacting with chloroformates, to give N-alkoxycarbonyl derivatives. - Resistant to enzymes. - Bioreversible. - Increased lipophilicity.	
7.	Cyclic produgs of peptides. - Resistant to peptidic enzymes. - Bioreversible under esterase activity. - Increased lipophilicity. - Simultaneous masking of amino and carboxyl groups of peptide. - Reduction in intermolecular hydrogen bonding potential of peptide.	 acyloxy alkoxy-based cyclic prodrug coumarinic acid-based prodrug phenyl propionic acid based prodrug
8.	Modification of terminal carboxylic group of peptide to make oxazolidinone derivative. - Resistant to carboxypeptidase. - Bioreversible. - Decreased polarity and increased lipophilicity.	

Chart 1



				Oral activity in mice: ID_{50} (mg/kg) mean±SEM (n)
Parent drug, **I**:	R_1=H,	R_2=H	R_3=H	8.5±0.8 (12)
Single prodrug, **II**:	R_1=H	R_2=H	R_3=C_2H_5	6.9±1.1 (4)
Single prodrug, **III**:	R_1=C_2H_5OCO	R_2=H	R_3=H	10.7±2.8 (2)
Double prodrug, **IV**:	R_1=C_2H_5OCO	R_2=H	R_3=C_2H_5	1.4±0.4 (4)
Double prodrug, **V**:	R_1=OH	R_2=H	R_3=C_2H_5	2.6±0.7 (5)
Triple prodrug, **VI**:	R_1=C_2H_5OCO	R_2=CH_3CO	R_3=$CH_3CH_2CH_2$	0.9±0.3 (4)

suggested, e.g., Weller and co-workers[76] prepared prodrugs of Lamifiban (Ro 44−9883; **I**) by one modification (modification of either carboxyl or amidino group; **II, III**), two modifications (modification of both carboxyl and amidino groups; **IV, V**), and three modifications (modification of carboxyl, amidino, and phenyl hydroxyl groups; **VI**), Chart 1.

Triple prodrug (**VI**) was found to be more orally active (lower ID_{50}) in mice than double prodrugs (**IV, V**), which in turn showed higher oral activity than single prodrug (**II, III**). In a recent review Wang et al.[77] discussed various prodrug-based strategies to improve bioavailability of peptidomimetic RGD (Arg-Gly-Asp) analogues.

The prodrug approach can also be used to intercept with the P-glycoprotein-mediated efflux of PP drugs. However, to modify PP drugs to reduce or prevent their substrate activity for efflux systems it is important to know the structural features that influence efflux-mediated transport for PP drugs.[78] Broad substrate specificities shown by efflux systems make it difficult to identify the suitable chemical modifications for altering susceptibility characteristics of PP drugs toward efflux systems. Instead, use of P-glycoprotein inhibitors, such as the *R*-isomer of verapamil, nonimmunosuppressive analogues of cyclosporin D (SDZ PSC833) and LY335979 as adjuvants appears to be a more realistic approach to help improve oral absorption of PP drugs that are substrates for intestinal efflux systems.[58]

One of the most important features of a prodrug is the ability to be converted quantitatively to the parent peptide in vivo by a spontaneous or unspecified plasma enzyme-catalyzed reaction after their absorption.[79] Modified peptides that lack bioreversibility are considered to be new peptides rather than prodrugs, and the approach is known as an analogue approach. Chemical modification of proteins by succinylation, acylation, guanidation, modification of amide bonds, and deamination conjugation with polymers such as dextran, albumin, DL-poly(amino acid), poly(vinylpyrrolidone), and poly(ethylene glycol) have been tried to increase the blood circulating life and/or reduce immunogenicity.[80−82] Toth and co-workers reported modification of N- and C-termini of TT-232, a tumor-selective somatostatin analogue, to improve its stability and bioavailability. They prepared lipoamino acid and liposaccharide conjugates of TT-232, which resulted in amphipathic surfactant molecules with retained activity and improved transport across Caco-2 cell monolayers.[83] In an attempt to use the lymphatic absorption pathway and thereby bypass hepatic first pass metabolism, Delie et al.[84,85] prepared the diglyceride prodrug of a pentapeptide rennin inhibitor SR 42128. Conjugation of pentapeptide drug to 2-position of 1,3-diglyceride resulted in a prodrug of increased lipophilicity and better stability to degradation by proteases and peptidases (intestinal juice and α-chymotrypsin). However, lymphatic uptake of prodrug on oral administration to rats could not be established. Various approaches for derivatization of PPs and recently published studies highlighting use of the prodrug strategy to improve *peroral* bioavailability of PP drugs are summarized in Tables 3 and 4. In addition to altering the physicochemical properties of PP drugs to improve their transmembrane passive permeability and stability to enzymatic degradation, the prodrug approach has been used to enhance substrate property of PP drugs to carrier-mediated active transport mechanisms, which is discussed later in the review.

1. Pegnology

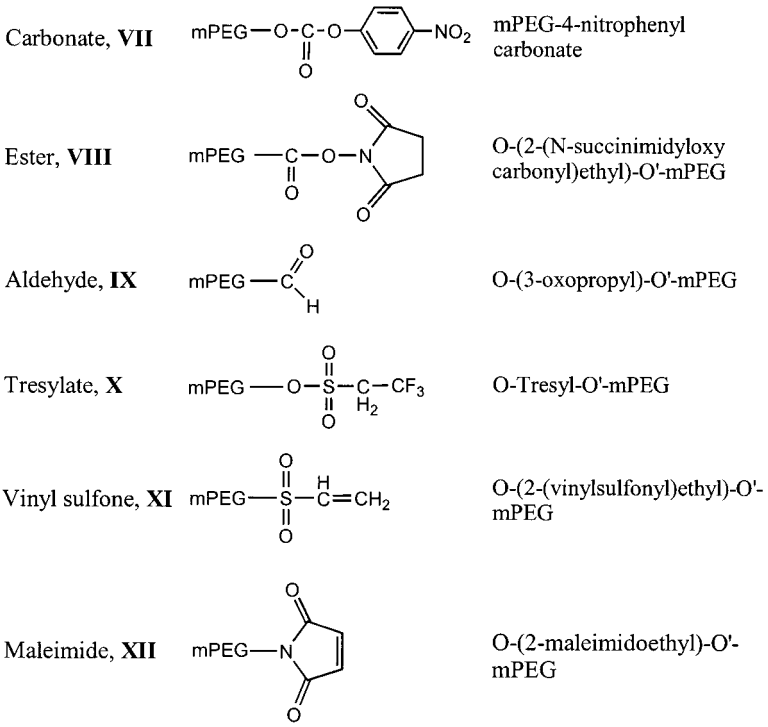
Therapeutic proteins have been coupled to various polymers so as to reduce their immunogenic response, increase resistance to enzymatic degradation, and prolong their half-life. Oral absorption of PP drugs has been achieved by chemically changing the protein or peptide by covalent addition of the polymers composed of water- and fat-soluble elements. Polymers such as poly(ethylene glycol) (PEG), dextran, albumin, and poly(vinylpyrrolidone) have been studied as protein carriers.[3] Modification of proteins with PEG is known as pegnology or pegylation and has been shown to improve biopharmaceutical and clinical properties (including enhanced solubility, sustained absorption, reduced immunogenicity and pro-

Table 4. Some of the Prodrugs/Analogues of Proteins and Peptides Screened for *Peroral* Bioavailability[a]

biologically active species	prodrug/analogue	results	ref
DDAVP	pivalate, *n*-hexanoyl and *n*-octanoyl esters of the tyrosine phenolic group in dDAVP	sterically hindered pivalate ester was more stable to enzymatic degradation	306
Insulin	Palins-1 Palins-2	increased plasma radioactivity on administration in polyoxyethylene hydrogenated castor oil (HCO60)	53,307
Insulin	glycosylated insulin	provide affinity to transport carriers	165
Leu-enkephalin	four different 4-imidazolidinones of Leu-enkephalin	prodrugs were found to be stable to angiotensin-converting enzymes and aminopeptidases N enzymes that are responsible for degradation of Leu-enkephalin at the BBB and in plasma	308
Leu-enkephalin and its metabolically stable analogue DADLE	phenylpropionic acid-based and acyloxyalkoxy-based esterase-sensitive cyclic prodrugs	prodrugs were found to be more lipophilic, more stable against peptidase metabolism, and many fold better permeating across Caco-2 cell monolayers than their respective linear opioids; chemical stability studies revealed stoichiometric conversion of prodrugs to the corresponding peptides; however, for aycloxyalkoxy-based prodrugs, apical to basolateral permeability was lower than that of DADLE and also lower than their permeability in basolateral to apical direction due to polarized efflux system	68−70,75
Leu-enkephalins	4-imidazolidinone derivatives with acetaldehyde, acetone, and cyclopentanone	completely inert toward aminopeptidases and ∝-chymotrypsin, decomposes at pH 7.4 and 37 °C with half-lives of 30, 10.9, and 3.1 h, respectively, lipophilicity of prodrugs was increased; however, it could be easily degraded by carboxypeptidase A	309
MDP	MTP−PE	MTP−PE has immunostimulant effects similar to those of natural muramyl dipeptide and has a longer half-life in plasma and lower toxicity	310
model hexapeptide	acyloxyalkoxycarbamate-based and phenylpropionic acid-based esterase-sensitive cyclic prodrugs	cyclic prodrugs degraded to linear hexapeptide in various biological media due to esterase activity; cyclic prodrugs were more stable to peptidase metabolism and more permeable when applied to apical side of Caco-2 cell monolayers	71−74
N-acetyl-L-phenylalaninamide	peptidyl-α-hydroxy glycin derivatives	derivatives were found to be completely resistant to hydrolysis by α-chymotrypsin	311,312
RGD analogues	Coumarine-based cyclic prodrugs	prodrugs of RGD analogues showed enhanced membrane interaction potentials (determined from their partitioning between 10 mM phosphate buffer, pH 7.4/acetonitrile as various concentrations, and an immobilized artificial membrane) and intrinsic membrane permeabilities (determined using Caco-2 cell monolayers); prodrugs were found to undergo esterase-catalyzed release of RGD analogues in the presence of porcine lever esterase; prodrug of compound MK-383 showed significant and prolonged antiplatelet activity (determined ex vivo after oral administration to a dog) in contrast to parent compound	313,314
TG	acetyl-tetragastrin caproyl-tetragastrin	significant increase in gastric acid secretion observed in comparison with TG	315
TG	acyl TG	stability of TG in plasma and intestine was improved by conjugation of the acyl group	316
TRH	γ-butyrolactone-γ-carbonyl-L-histidyl-L-prolinamide citrate	absorbed from all parts of small intestine in a nonsaturable fashion, 2−5 times greater CNS action than TRH	317
TRH	lauroyl-TRH	CNS activity and endocrine activity slightly reduced to 81% and 64%, respectively, to that of parent, but TRH was found to be more stable	67,318,319
TRH	*N*-alkoxycarbonyl prodrug derivatives (formed by N-acylating the imidazole group of histidine residue) *N*-α-phthalidyl derivative (formed by attachment of a phthalidyl group to imidazole group of histidine residue)	derivatives were resistant to cleavage by TRH-degrading serum enzyme, bioreversible, and with increased lipophilicity and half-life relative to TRH; prodrugs showed improved skin penetration; however, they were not useful to improve intestinal absorption	320,321, 322
tripeptide (Ac−Phe-Phe-Phe-NH_2)	series of four peptide analogues with increasing number of *N*-methylated amine bonds	peptide containing all N-methylated amide bonds displayed the highest intestinal permeability; authors hypothesized that decreasing the desolvation energy that is required to remove a peptide from aqueous environment to the lipid environment of epithelial cell membrane increases intestinal permeability	64

[a] TRH: thyrotropin releasing hormone; Palins-1: B1-monopalmitoyl insulin; Palins-2: B1, B29-dipalmitoyl insulin; TG: Tetragastrin; dDAVP: Desmopressin; MDP: muramyl dipeptide; MTP−PE: muramyl tripeptide−phosphatidylethanolamine.

Chart 2



teolysis, reduced renal clearance, decreased dosing requirements due to increased circulation time, optimized distribution, and reduced toxicity) of a number of proteins such as granulocyte macrophage colony stimulating factor (G-CSF), interleukin-2, tumor necrosis factor (TNF), and interleukin-6.[86,87] The success of PEG-modified proteins is because of its nontoxic, nonimmunogenic, and highly water-soluble nature. Additionally, PEGs are readily cleared from the body and are approved for use in foods, cosmetics, and prescription medicines by the US-FDA.[3,88]

The complex formation is a two-step procedure which involves the following. (i) The first step is preparing an activated PEG with a reactive functional group: Two terminal groups of PEG are chemically activated by means of substitution with electrophilic functional groups to give active carbonate (**VII**), ester (**VIII**), aldehyde (**IX**), tresylate (**X**), vinyl sulfone (**XI**), or maleimide (**XII**) groups on the PEG moiety. To prevent the possibility of cross-linking, it is preferred to use a monofunctional PEG molecule, such as monoalkoxy PEG (mostly monomethoxy PEG, mPEG), that can be obtained by converting one of the two hydroxyl groups to a methoxy or an alkoxy group. Various modifications can be done to achieve PEGs of many different architectures, such as branched activated PEG (**XIII**), forked activated PEG (**XIV**, **XV**), multiarm PEG (**XVI**), etc.

$$\underset{\text{monomethoxy PEG}}{CH_3O(CH_2CH_2O)_n-CH_2CH_2\cdot OH} + \xrightarrow{\text{activating group}} \underset{\text{activated PEG with reactive functional group X}}{CH_3O(CH_2CH_2O)_n-CH_2CH_2-X} \leftrightarrow mPEG-X$$

Examples of activated PEG with different reactive functional groups are shown in Chart 2. Examples of activated PEG with different architectures are shown in Chart 3.

(ii) The second step is anchoring activated PEG to amino or thiol groups on native protein, leading to formation of amide, urethane (carbamate), or thioester linkages. PEG−carbonates, −esters, and −aldehydes yield amine-specific pegylation by modifying PP amino groups (such as N-terminal or lysine amino groups), producing stable urethane or amide linkages, whereas PEG−maleimides give thiol-specific pegylation, Chart 4.

The overall performance of pegylated protein is governed by a number of factors such as stability of linkage, degree of cross-linking, size of PEG, structure of PEG (linear or branched), and number of attachments (linkage at a single site or multiple sites of native proteins). It has been shown that amide and urethane linkages of PEG active ester or carbonate with lysine in a protein are more stable as compared to those formed with histidine residues.[89] Similarly, complexes formed by single-site attachment between a branched PEG moiety and protein are found to give a better pharmacological profile of native protein, whereas multiple-site linkages using small linear chain PEGs or linkages with large PEGs may result in loss of bioactivity of protein due to the possibility of linkage at or near receptor binding domains.[86,88] The branched structure of the PEG moiety results in a relatively large molecular volume so that the advantage of PEG attachment can be obtained without many points of attachment. However, forked PEGs have the advantage of placing two reactive groups at a precise distance apart. Decreased immunogenicity, increased half-life, and resistance to proteolysis of PEG-coupled proteins as compared to

Chart 3

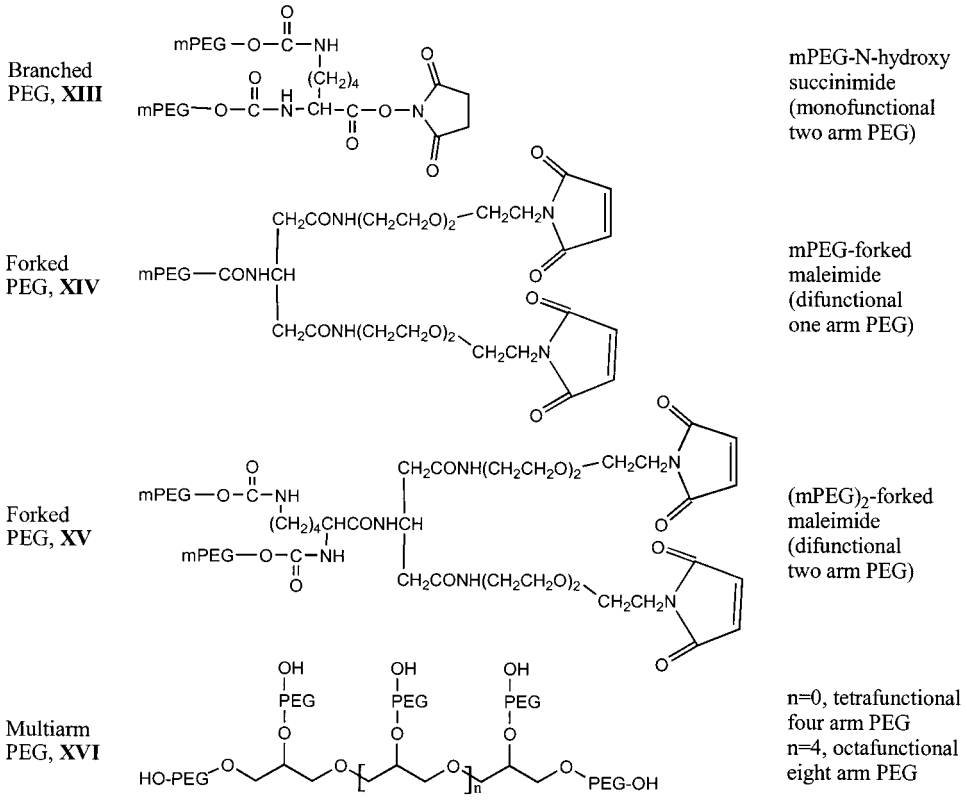
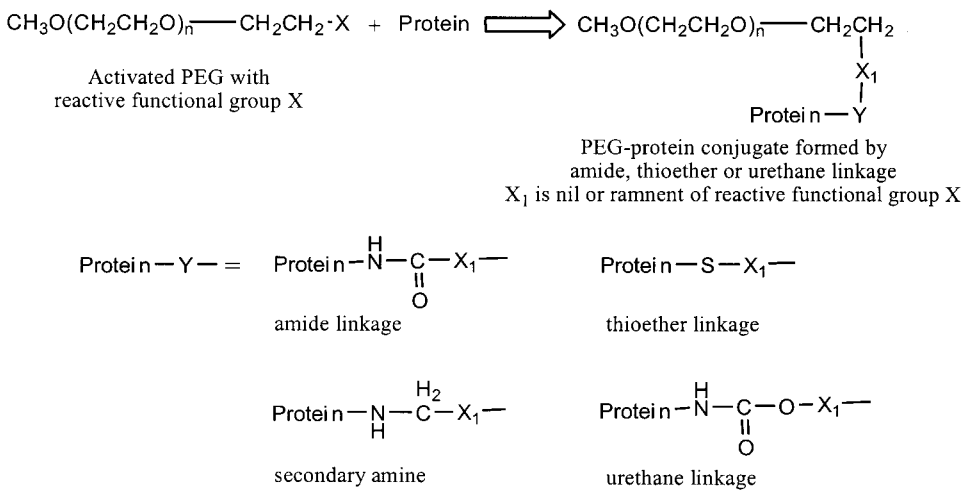


Chart 4



native proteins are believed to be the result of steric hindrance and extra weight provided by PEG.[87,90] Fuke and co-workers[91] prepared one-branched (PEG_1), two-branched (PEG_2), and three-branched (PEG_3) PEG derivatives and coupled them to trypsin. It was found that substrate activity of PEG−trypsin to digestion by pepsin decreased in the order of unmodified trypsin > PEG_1 > PEG_2 > PEG_3, suggesting the increasing steric hindrance effect of PEG derivatives with increasing number of PEG chains.

Until recently, Pegademase bovine or PEG−adenosine deaminase (Adagen) was the only PEG−protein complex approved by the US-FDA as replacement therapy for adenosine deaminase deficiency in patients with severe combined immunodeficiency disease. There are now other products approved by EU and US-FDA that have been developed using this approach: Pegintron (pegylated interferon alpha-2b) and Pegasys (pegylated interferon alpha-2a) for treatment of chronic hepatitis C and Oncospar (pegylated asparaginase) for the treatment of acute lymphoblastic leukemia are the new additions.[88,92] PEG-modified proteins such as PEG−L-asparaginase, PEG−superoxide dismutase, PEG−uricase, and PEG−interleukin-2 are some of the classical examples of prodrugs, also termed as altered chemical entities, which have been promoted for clinical trials.[3]

The successful development of PEG-coupled proteins has shown the possibility of improving pharmacokinetic and pharmacodynamic parameters of native proteins, and although much of the success has been limited to parenteral application, pegylation technology is now being explored for developing orally effective molecules. PEG−insulin (composed of the

protein plus small amphiphilic polymer) is one such altered chemical entity that has been developed by Protein Delivery, Inc. (now Nobex Corporation) for *peroral* administration. PEG−G-CSF and PEG−INFcon are two other pegylated derivatives of G-CSF and consensus-interferon, respectively, developed by Nobex Corporation for *peroral* delivery. There are now different therapeutic proteins (e.g., calcitonin, parathyroid hormone, and enkephalin) that are presently under development for their *peroral* administration using the amphiphilic polymer conjugation approach.

B. Physical Barriers to Absorption and Absorption Enhancers

Transepithelial pathways available for molecular transportation from intestinal lumen to bloodstream can be classified as follows: (i) Transcellular passive transport; (ii) Transcellular carrier-mediated active or facilitated transport; (iii) Paracellular transport.

Physical barriers, which serve as a defense for the body against permeation of xenobiotics upon oral ingestion, include GIT epithelial lining with thick and viscous pellicle of mucus. The epithelial lining of GIT consists of a tightly bound single layer of columnar epithelial cells supported by *lamina propria* and *muscularis mucosa*. Three types of the junctions exist in the epithelial lining: desmosomes or *zonulae adherens*, tight junctions (TJs) or *zonulae occludens*, and gap junctions.[93] TJs provide the mechanical strength to cell lining by holding cells tightly bound to one another and constitute the major barrier for large molecular weight drug permeation between the cells (paracellular transport). The lipid nature of absorptive epithelial cell layer functions as a barrier for absorption of hydrophilic drugs through the epithelial cells (transcellular transport).

Drugs depending on their physicochemical properties are absorbed through these absorption portals. However, owing to their large molecular weight and hydrophilic nature, absorption of PP drugs through transcellular as well as paracellular route is severely restricted and is one of the major reasons for their poor bioavailability. It has been shown that very small fractions of luminally administered PPs can cross the small intestine in an intact form.[94,95] Ziv and Bandayan[96] performed morphological and electron microscopic immunochemical studies to show that insulin can be absorbed in an intact form from intestinal lumen through transcytotic pathway. Taking insulin as an example, they suggested that the mechanism for transcellular absorption of PPs involves, first, binding of PPs to specific receptors on apical plasma membrane[97,98] followed by internalization through deep invaginations of the luminal plasma membrane and vesicular structures.[96,99] The PP is then transferred to basolateral membrane of enterocytes and released into interstitial spaces without any degradation. However, this transfer is characterized by low transport rates and must be enhanced to allow absorption of PP drugs in significant amounts.

A possible approach to improve the bioavailability of PP drugs is to modify the barrier properties of different routes of absorption so as to make the absorption surface more permeable to these drugs. This can be achieved by using various agents classified as absorption enhancers. Absorption enhancers are defined as "formulation components that act upon different drug absorption pathways with a definite mechanism of action to improve the permeation of poorly permeable drugs". Most absorption enhancers disrupt the intestinal barrier. However, because of the possibility of toxicological manifestations, rapid reversibility and the transient nature of damage caused by absorption enhancers are the keys to their acceptability. Extended disruption of the barrier function of GI cell layer may result in loss of control over water and ion movement, consequently, resulting in diarrhea (as observed in number of intestinal pathologies in which barrier properties have been compromised), and at the same time it might open a window for nonspecific absorption of toxins or other chemicals that could lead to other side effects.[100,101]

1. Modulation of Transcellular and Paracellular Absorption Pathways

Drug absorption through a transcellular pathway is dependent on lipophilicity, molecular size, and charge of the molecules, of which an optimal lipophilicity is considered to be the most important attribute for the drugs crossing through this route. However, it is now believed that lipid solubility, as expressed by the octanol−water partition coefficient, does not influence the membrane permeability of PPs to the same extent as for small organic molecules. Instead, hydrogen-bonding potential is considered to be a better parameter to correlate to the lipophilicity and, hence, transmembrane permeability of PPs.[61]

With the exception of those molecules that are transported by active or facilitated mechanisms, the absorption of hydrophilic molecules is mainly limited to a paracellular pathway.[15] This route takes advantage of the leakiness of cell to cell junction, TJ, and is mainly open to ions and small molecules with molecule radii <11 Å. Several peptide drugs such as octreotide, vasopressin analogue desmopressin (dDAVP), and thyrotropin releasing hormone (TRH) are believed to be absorbed by this route.[61] It is now well established that TJ are dynamic structures that adapt to a variety of developmental, physiological, and pathological circumstances[102−107] and thus render themselves modifiable by absorption enhancers. PZ-peptide, a pentapeptide with a *p*-phenylazo(*p*-benzyloxycarbonyl) group attached at the amino terminus of the peptide, was reported a few years back to cause transient TJ expansion and facilitate paracellular transport of drugs.[108] Studies with fluorescent marker proteins have shown PZ-peptide to assist the transport of proteins up to 4000 to 5000 Da in size, suggesting the possibility of using it to enhance the delivery of proteins such as insulin, calcitonin, glucagon, dDAVP, and enkephalins.

Various authors have discussed the mechanisms involved in the improvement of intestinal permeation by absorption enhancers through these pathways.[93,109−111] These mechanisms, in general, involve the following. (i) Interactions of absorption enhancers

with membrane lipid/protein, which leads to membrane perturbation followed by an increase in permeability, e.g., naturally occurring bile acids, bile acid derivatives, surfactants, middle chain fatty acids, salicylic acid, and acyl carnitine. Additionally, bile salts have also been found to inhibit proteolytic enzymes.[112,113] Bai used different bile salts and showed their inhibitory activity on various brush-border peptidases and cytosolic insulin-degrading enzymes at low salt concentrations.[113,114] (ii) Increased disorder of membrane status by decrease in membrane nonprotein thiol, e.g., diethyl maleate, salicylic acid. (iii) Capability of bile salts to form complexes with calcium is shown to be responsible for opening of transcellular TJ and improve FITC-dextran permeation across Caco-2 cell monolayers through paracellular route.

In a report published recently, Hayashi et al. described the action mechanisms of absorption enhancers to be either physiological or pathological based on the recoverability of the membrane function.[115] The influence of various drug transport enhancers (sodium caprate, acylcarnitines, and organic acids) on the paracellular route of absorption (i.e., TJ), their action mechanisms, and recovery of membrane dysfunction caused by these agents was studied. Fasano described the role of *zona occludin* proteins in assembly and regulation of TJ and explained that *zona occludin* toxin (ZOT) can be applied to enhance intestinal paracellular absorption of normally unabsorbable macromolecules such as insulin and immunoglobulins.[116,117] ZOT acts specifically on the actin filaments of TJ in jejunum and ilium to cause safe, reversible, time- and dose-dependent regulation of paracellular pathway.[118] This controlled permeation enhancement is considered to be better than nonspecific disruption caused by fatty acids and bile salts. The increase in *peroral* bioavailability of PP drugs by using absorption enhancers has been exhaustively reviewed and enlisted in the past by different authors.[119−121]

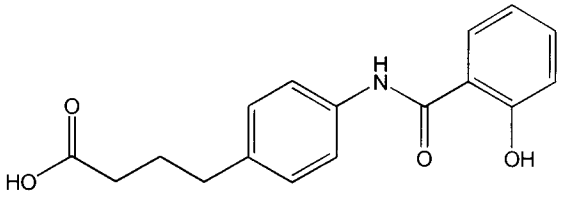
Attempts to find ways to increase drug absorption by altering the barrier properties of GI absorption surface have been hampered by toxic effects induced by the potential absorption enhancing agents tested so far.[122,123] In the case of calcium chelators, Ca^{2+} depletion induces global changes in the cells, including disruption of actin filaments, disruption of adherent junctions, and diminished cell adhesion.[124] Similarly, toxicological manifestations associated with the use of bile acids as absorption enhancers are a subject of major concern.[125−127] On the basis of the results of a toxicity assessment of bile acids, Michael et al. recommended that chenodeoxycholic acid, despite its very good absorption enhancing effects demonstrated for octreotide and dDAVP, cannot be used for chronic administration.[128] The action of PZ-peptide on TJ opening has been found to involve its interaction with sodium channels (Na^+/K^+ exchangers and amiloride-sensitive Na^+ channels) and stimulate Na^+ entry into epithelial cells, thereby limiting its use as penetration enhancer.[129]

The success of developing peptide formulations using absorption enhancers hinges on a balance of absorption enhancement activity and degree of cell structure perturbation. Hence, absorption enhancers of desired properties such as peptide compatibility, rapid response at low to moderate concentration, rapidly reversible effect on absorption mucosa, specific permeability enhancement effect, well-defined mechanism of action, suitable physicochemical properties for easy formulation, easy availability on commercial scales, and nontoxicity are present day's demand. While searching for such ideal absorption enhancers, chitosan has been found to be of value due to its ability to open TJ, biocompatibility, biodegradability, natural origin, and absence of systemic and local toxicity.[130,131] However, lack of functionality at neutral pH values due to aggregation and precipitation has limited its use as penetration enhancer. *N*-Trimethyl chitosan chloride, a chitosan derivative with different degrees of trimethyl substitution, has been shown recently to enhance intestinal absorption of peptide drug buserelin in neutral pH values.[132] Nearly all studies with enhancer formulations for peptide delivery in animals have shown that at least transient changes in membrane integrity occur with formulations showing significant activity. In a study using octreotide, dDAVP (model peptides), and FITC-dextran (paracellular marker), Michael et al. showed that the absorption efficiency across Caco-2 cell monolayers as well as in rats could be increased in the presence of chenodeoxycholic acid, cholyltaurine, and cholylsarcosine.[128] However, absorption enhancement and their cytotoxic potentials across the Caco-2 cell layer were found to be in the same rank order of chenodeoxycholic acid > cholyltaurine > cholylsarcosine, indicating that the cell impairment effect is correlated to the absorption enhancing capability of the bile acids. Cholylsarcosine, a relatively weaker biological enhancer, was suggested to be safer and a preferable choice due to its lower cytotoxicity. Morishita et al. showed that docosahexaenoic acid and eicosapentaenoic acid, long chain polyunsaturated fatty acids, incorporated in water-in-oil-in-water multiple insulin emulsion strongly enhance intestinal absorption of insulin.[133] The absorption enhancing effect of two fatty acids was insulin dose dependent, and their use did not cause any significant membrane damage. However, use of middle chain fatty acid salts (sodium laurate and sodium caprate), which primarily increase paracellular transport, has been shown to be associated with serious side effects in Caco-2 monolayers.[134] Although GIT has been shown to exhibit a dramatic ability to restore normal cellular architecture and function following fairly severe perturbations also, most reports of enhancer formulations in the literature do not address what types of tissue responses, particularly reversibility, are elicited by chronic, repeated administrations.

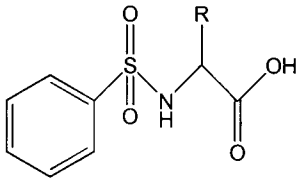
Another approach that appears to increase transcellular intestinal absorption without damaging the epithelium and thus has generated lot of interest in the past few years is described here:

a. Complex-Forming Delivery Agents. PP drug molecules can exist in different conformations under physiological conditions, and some of these conformations can be transported intact across the cell mem-

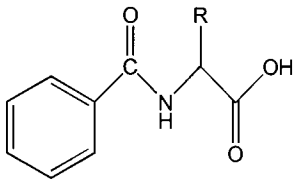
Chart 5



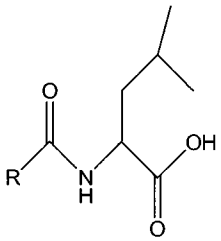
XVII



XVIII



XIX



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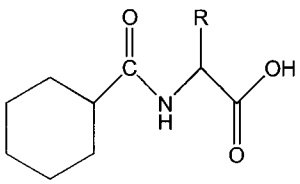
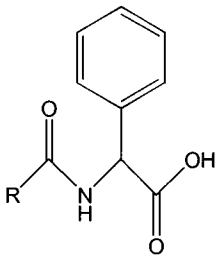


XXI

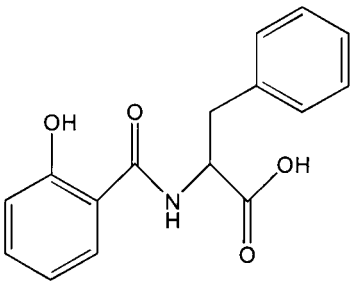


XXII

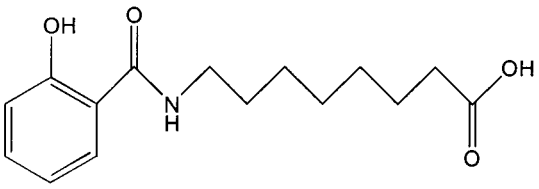
brane.[135] A research team at Emisphere Technologies, Inc., New York, has been working on an approach of improving stability of conformations of PP drugs (those conformations which favor permeation across cell membrane) by means of using suitable chemical agents so as to improve their transepithelial transport across GI epithelium. Leone-Bay and co-workers (Emisphere Technologies, Inc.) recently reported the use of N-acetylated, non-α, aromatic amino acids and N-acylated α-amino acids to promote *peroral* delivery of variety of therapeutic proteins such as salmon calcitonin (sCT), interferon-α, insulin, and recombinant human growth hormone (rhGH). Of the 70 compounds tested to facilitate the *peroral* absorption of rhGH in rats, 4-(4-(2-hydroxybenzoyl)aminophenyl)butyric acid (SABA, **XVII**) was found to be most efficacious. *Peroral* administration of a single dose of rhGH in combination with SABA was shown to give a mean peak serum concentration of 55ng/mL of rhGH in cynomolgus monkeys, which otherwise in the absence of SABA or administration of SABA alone remained below measurable levels of rhGH.[136,137] Similarly, some other test agents viz. *N*-(phenylsulfonyl)-α-amino acids (**XVIII**), *N*-benzoyl-α-amino acids (**XIX**), derivatized leucines (**XX**), *N*-cyclohexanoylamino acids (**XXI**), and derivatized phenylglycines (**XXII**) have been found to increase *peroral* absorption of sCT and interferon-α in rats and cynomolgus monkeys, Chart 5.[138]

In an attempt to elucidate the mechanism of activity of these agents, the Emisphere research group showed that enhanced drug permeation across the intestinal membrane is neither due to alteration in membrane structure (i.e., mucosal damage) nor a result of direct inhibition of physiological mechanisms of degradation.[136,138,139] Instead, it has been postulated that some type of noncovalent interaction between PP drug and delivery agent molecules may be responsible for efficient drug absorption through the intestinal mucosa.[140,141] In an experiment, Leone-Bay et al. found that at a constant dose of rhGH and delivery agent SABA (in terms of mg/kg body weight of rats), efficiency of drug delivery increased with decreased dose volume, suggesting the effect to be due to increased intermolecular associations at high concentrations, i.e., low dose volume.[136] Also, from structure−activity relationships, it was found that among the structurally related groups of compounds, more lipophilic compounds (lipophilicity determined as log k' values using immobilized artificial membrane chromatography or HPLC retention time as a measure of logP) had a better ability to promote protein (rhGH, sCT) absorption.[136,138] However, no such correlation could be established in the case of interferon-α, probably due to its large and complex structure, which suggests that there are other influential factors that are crucial for an optimal interaction of delivery agent with drug and hence for their drug delivery activity. In a recent study, Milstein and co-workers[139] showed that noncovalent interactions of delivery agents and proteins cause temporary stabilization of partially unfolded conformations of proteins exposing their hydrophobic side chains. The altered lipid solubility of stabilized conformations, as a result of exposed hydrophobic side chains, permits them to gain access to pores of integral membrane transporter and thus are more absorbable through lipid bilayers.[142,143] The delivery agent−protein combination, which is held together by weak noncovalent intermolecular forces, gets separated after membrane transport as a result of dilution. This ensures reversion of protein into its biologically effective conformation from partially unfolded conformations. It has been established that the biological activity of insulin remained unaffected as a consequence of administration with *N*-(2-hydroxybenzoyl)phenylalanine (**XXIII**). Supporting this mechanism, Wu and Robinson used Caco-2 cell monolayers to show that interaction of human growth hormone with SABA (**XVII**) and *N*-(8-(2-hydroxybenzoyl)amino)caprylate (SNAC, **XXIV**) makes the protein a better substrate for P-glycoprotein (which is known to be overexpressed in apical surface of Caco-2 cells), thereby suggesting that the interaction causes the protein to be more lipophilic, Chart 6.[144]

Chart 6



XXIII



XXIV

The mechanism of action of these agents is still not clear and efforts are being made to explore the same. Various absorption pathways that may be involved in the permeability enhancement effects shown by these agents include (Figure 2) the following:[145] (i) Transcellular transport involving partitioning of a therapeutic into the plasma membrane and the subsequent diffusion of the molecule around the perimeter of the cell through apical membrane; (ii) Transcytotic transport by pinocytosis or receptor-mediated endocytosis; (iii) Paracellular transport process involving diffusion of a molecule along a hydrophilic pathway between cells through TJ.

Stoll and co-workers correlated membrane permeability (K_p) to different transport parameters related to these transport routes in eq 1[145]

$$K_p = (A^{mv}/A^{pmv})\left(\frac{k \cdot D_L}{l} + k_t + k_p\right) \qquad (1)$$

where k = partition coefficient of therapeutic into apical epithelial membrane, D_L = lateral diffusion coefficient of therapeutic through apical epithelial membrane, k_p = paracellular rate constant between epithelial cells (i.e., through hydrophilic pores), k_t = transcytotic rate constant, A^{mv}/A^{pmv} = surface area expansion provided by the microvilli, and l = transcellular passive diffusion length.

The magnitude of each transport parameter can be expressed as the sum of the contributions from molecules in the partially unfolded state (I) and the native state (N) as

$$M = \alpha M^I + (1 - \alpha)M^N \qquad (2)$$

where M is a generic transport parameter dependent on the particular absorption pathway under consideration and α represents the fraction of protein molecules stabilized in the intermediate state at equilibrium for the pH range encountered near the apical membrane. Changes in conformation of protein from native state to partially unfolded state can

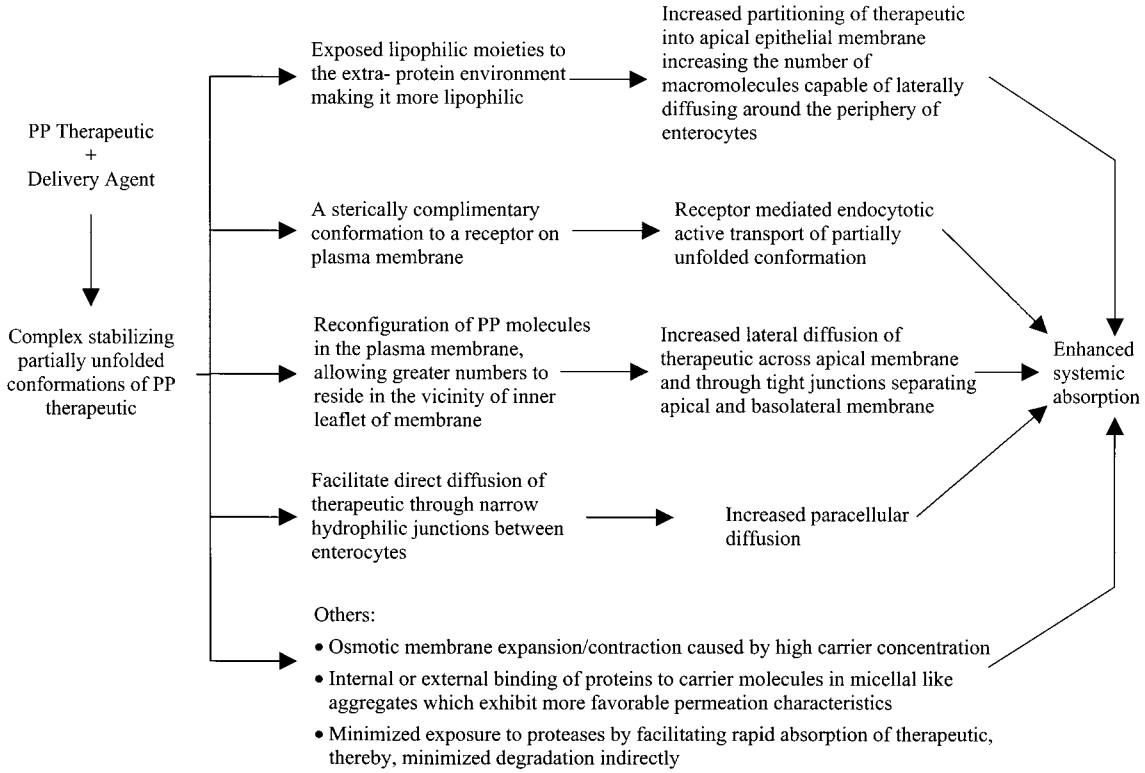


Figure 2. Possible mechanisms of *peroral* absorption enhancement of PP therapeutics by novel delivery agents.

influence the magnitude of these transport parameters, thereby resulting in enhanced membrane permeability[145] (Figure 2).

2. Carrier-Mediated Transport

It has long been believed that synthetic drugs are absorbed through the GIT epithelium by simple diffusion mechanism. However, there are direct and indirect evidences for participation of carrier-mediated active and facilitated membrane transport mechanisms, where several hydrophilic compounds seem to be absorbed efficiently via such specialized transporters. Electrochemical gradients of Na^+ and H^+ across enterocytes provide the driving force for carrier-mediated transport. The Na^+/K^+-ATPase (present on the basolateral membrane) and Na^+/H^+-exchanger (present on luminal surface) are responsible for lower intracellular Na^+- and H^+-ion concentrations in intestinal epithelial cells, respectively, which as a consequence results in inwardly directed Na^+- and H^+-gradients. Transport systems are thus Na^+-gradient dependent (e.g., amino acid, bile acid, phosphate transporters) or H^+-gradient dependent (e.g., oligopeptide, short chain fatty acid transporters) based on the nature of the ionic (Na^+ or H^+) co-transport energizing the transport system. Various transporters that are present in the GI epithelial cells can be classified as follows:[146,147] (i) transporters for amino acids and oligopeptides, (ii) bile acid transporters, (iii) water-soluble vitamin transport systems, (iv) carbohydrate transporters, (v) monocarboxilic acid transporters, and (vi) phosphate transporters.

Utilization of the intestinal epithelial transporters to facilitate the absorption of appropriately modified drugs is an attractive strategy for improving the bioavailability of poorly absorbed drugs such as PPs. Among the transport pathways in the enterocyte, the largest molecules taken up by a carrier-mediated mechanism are (1) bile acids with M_r of 537 for taurocholate, taken up by a Na^+-dependent uptake mechanism;[148,149] (2) *perorally* active α-amino-*β*-lactam antibiotics with a M_r of 347 for cephalexin, taken up by a H^+-dependent transport system for di- and tripeptides formed from ingested proteins;[150−152] (3) folate with a M_r of 441, taken up by a carrier-mediated uptake mechanism.[153]

The intestinal transport systems for bile acids and oligopeptides have transport capacities greater than 10 g/day, making them attractive opportunities which can be exploited for PP drug delivery.[147] An H^+-coupled peptide transporter protein, PepT1 has been cloned and its role in improving the absorption of small polar drugs by means of designing suitable prodrugs has been described.[146,154] Despite a large number of compounds known to show affinity for intestinal peptide transport systems (i.e., broad substrate specificity), the structure−transport relationships are still not clearly understood. However, few structural features such as a free terminal carboxyl group (i.e., group capable of hydrogen bond formation), amino group, or weakly basic group at the N-terminus, preferably the L-configuration of C-terminal amino acid, and overall charge of less than two positive units are considered important to impart substrate specificity to the molecular structures for peptide transporters.[155] Yang et al.[156] recently discussed various peptidomimetic drugs that are substrates for intestinal peptide transporters and compared their structural features and substrate specificities.[156] Kramer et al., however, reported that the H^+/oligopeptide transport system might not be well suited as a drug delivery shuttle system to improve the intestinal absorption of peptides due to rapid hydrolysis of the peptides by brush-border and intracellular hydrolyzing peptidases.[147]

The ileal Na^+/bile acid transport system has also been explored to enhance intestinal peptide absorption. There are certain structural elements that have been identified to be necessary for molecular recognition of a bile acid by the Na^+/bile acid transport system.[148,157,158] A series of small, linear model peptides of chain length up to 10 amino acids were covalently coupled to the 3-position of a modified bile acid to give peptide−bile acid conjugates which were found to be significantly less susceptible to hydrolysis in comparison to cephalexin conjugates.[147] Conjugation of peptides with bile acid seemed to prevent the access of conjugates to the brush-border peptidases, thus escaping the usual transport and metabolic pathways for peptides. It was found that bile acid conjugates of fluorescent-labeled peptides were transported in intact form from the intestinal lumen into bile, whereas the corresponding parent peptides could not be detected in the bile, indicating superior intestinal absorption of conjugates over parent peptides. The shape of the compound attached to the bile acids and site of attachment of peptide to bile acid are the factors reported to be important from drug delivery point of view. Compared to linear peptides, globular and rigid molecules of similar molecular mass have been found to show significantly lower absorption rates, indicating a diameter limitation for intestinal uptake of bile acid−drug conjugates. The transporter molecules for bile acids are located in the terminal ileum region of GIT, and hence, a peptide−bile acid conjugate has to cross the 'unfriendly' milieu in the entire length of the small intestine to reach terminal ileum before absorption. In contrast, the H^+/oligopeptide transport system is expressed in the entire small intestine and a peptide−ligand conjugate for the H^+/oligopeptide transporter would most likely be absorbed in the proximal GIT without prolonged exposure to intestinal conditions and thus can be more useful than bile acid carrier-mediated uptake.

Recently, it has been shown that the carrier-mediated uptake mechanism of vitamin B_{12} can be used to co-transport PPs bound to vitamin B_{12} from the intestine following *peroral* administration.[159−163] Habberfield et al. conjugated two recombinant human proteins, G-CSF and erythropoietin, to vitamin B_{12} and studied the uptake and transport of conjugates in vitro (across Caco-2 cell line) and in vivo (in male Sprague Dawley rats).[163] Both these conjugates were transported across Caco-2 cell monolayers in higher levels as compared to the unconjugated proteins. Also, serum concentrations of both conjugates in systemic circulation were found to be higher than

the unmodified proteins after duodenal instillation into rats.

A strategy for the enhancement of intestinal absorption by derivatization to monosaccharide analogues has also been applied to peptides. Although improved intestinal absorption has not yet been definitely ascribed to intestinal sugar transporters, the coupling of unstable peptides with sugars does improve both hydrolytic stability and membrane permeation.[164] Following modification of insulin with sugars, insulin was found to be more resistant to enzymatic hydrolysis and exhibited enhanced membrane permeation.[165] Thus, these natural transport pathways for nutrients and bile acids can be an option for *peroral* delivery of peptides and drugs. In addition, Russel-Jones[166] described the use of mucosal binding proteins, which can recognize surface determinants on the surface of epithelial cells, as carriers for *peroral* protein delivery. It has been shown that proteins such as lectins and many bacterial toxins can bind to specific residues on the intestinal epithelial cell surface and stimulate the uptake and transcytosis of these proteins across the epithelial cells.[167−169] On the basis of their transcytosis enhancing property, lectins act as transport molecules to cotransport PPs that are conjugated to them, across the intestine.

3. Mucolytic Agents

Apart from the absorption barrier based on GI epithelial permeability, the mucus layer can be regarded as another limiting factor being responsible for poor bioavailability of these therapeutic agents. Mucus is a thick, viscous, constantly changing mix of glycoproteins (mucins), enzymes, electrolytes, water, and exfoliated epithelial cells that covers the GI epithelial cell lining.[118,170] Mucus layer decreases the diffusion rate of drugs to reach the absorptive surface of epithelial cells. Although the effect is insignificant for low molecular weight hydrophilic drugs, glycoproteins (the gel forming and viscosity imparting component of mucus) present a more significant barrier to the diffusion of macromolecular PP drugs.[171] Studies focusing on this so-called 'diffusion barrier' have demonstrated that proteins of a molecular mass greater than approximately 5 kDa can hardly permeate this mucus layer.[172] Use of mucolytic agents, however, can strongly reduce this barrier, e.g., addition of 2% *N*-acetylcysteine led to an approximately 4-fold increase in the amount of a model protein, with a molecular mass of 12.4 kDa, that can permeate a porcine mucus layer within 5 h.[172] Moreover, in vivo studies focusing on the influence of the mucus gel layer on intestinal permeability demonstrated a significantly higher uptake of FITC-dextran 70 000 in rats due to the co-administration of *N*-acetylcysteine.[173]

On the basis of the mechanism of action, mucolytic agents can be generally divided into[174] (i) proteases cleaving the protein core of mucin glycoproteins, (ii) sulfhydryl compounds splitting mucoprotein disulfide linkages, and (iii) detergents breaking noncovalent bonds within the mucus.

The molecular size of comparatively large drugs such as polypeptides is mainly responsible for their very poor diffusion through the mucus layer. Bernkop-Schnurch et al., while describing a drug delivery system for trypsin and bromelin, showed that both proteolytic enzymes have strong mucolytic properties, which help the enzymes to permeate through mucus layer.[175] Use of various mucolytic agents such as protease (Pronase or papain) and thiols (dithiothreitol and *N*-acetylcysteine) leads to in vitro reduction in viscosity of porcine mucus; however, their in vivo application is strongly limited by the rapid degradation of the therapeutic polypeptide (insulin).[176] The authors suggested that sulfhydryl compounds might be more useful for (1) polypeptides exhibiting no cysteine moieties within their primary structure (e.g., cyclosporin, gonadotropin) and (2) protein drugs bearing disulfide bonds that are not accessible for thiols due to the conformation of the protein. Noninorganic detergents (Triton X-100 and Tween 20) were also shown to display weak mucolytic activity.

Strong liquefying action, without any degradation of the polypeptide drugs, is a prerequisite characteristic for mucolytic agents to help improve the bioavailability of *perorally* administered PPs. Glycosidases, which cleave glycosidic bonds within the mucus glycoprotein, might be promising candidates, although the agents that have been evaluated so far, such as α-amylase and β-amylase, have showed only a poor or no mucolytic effect.

C. Enzyme Barrier and Enzyme Inhibitors

Besides the barrier function of the mucus covering the GI epithelia, obstruction in absorption due to degradation during absorption, and hepatic first-pass metabolism, the rapid luminal enzymatic degradation can be regarded as a bottleneck accounting for low bioavailability of PPs. The enzymatic degradation of PP drugs is caused by luminally secreted, brush-border membrane-bound and cytosolic proteases and peptidases that are secreted or located throughout the length of GIT. It has been shown that pancreatic enzymes account for about 20% of enzymatic degradation of ingested proteins, and the rest of the degradation is caused by brush-border peptidases (active mainly against tri-, tetra-, and higher peptides, up to 10 amino acid residues) and cytosolic peptidases (active predominantly against dipeptides).[58,61] A promising strategy to overcome this so-called 'enzymatic barrier' represents the use of enzyme inhibitors, which has gained considerable interest in recent years. However, especially for PP drugs which are administered for a long duration, the co-administration of enzyme inhibitors remains questionable because of side effects caused by these agents and the interference with the regular digestion process of nutritive proteins.[177−180]

Use of enzyme inhibitory agents to *peroral* administration of PP drugs depends on the enzymatic barrier, which has to be considered both qualitatively and quantitatively. From the qualitative point of view, the enzymatic barrier is predetermined by the structure of the peptide or protein drug that should be *perorally* administered. The information on the specificity of proteases is therefore essential for the choice of enzyme inhibitor(s) in order to guarantee

Table 5. Various Luminally Secreted and Membrane-Bound Proteases and Their Inhibitors (modified from ref 208)

proteases	cofactor	inhibited by
		GI Luminally Secreted Proteases
pepsin		Pepsinostreptin, pepstatin, diazoacetyl-DL-norleucin methyl ester, valaminols, bovine uterine serpin, ovine uterine serpin, dioctylsodium sulfosuccinate
trypsin	calcium	Aprotinin, Bowman-Birk inhibitor, soyabean trypsin inhibitor, chicken ovomucoid, duck ovomucoid, chicken ovoinhibitor, human pancreatic trypsin inhibitor, camostat mesylate, flavonoid inhibitors, antipain, leupeptin, *p*-aminobenzamidine, organophosphorus inhibitors, polyacrylate derivatives, jack bean inhibitor
chymotrypsin	calcium	Aprotinin, Bowman-Birk inhibitor, soyabean trypsin inhibitor, chymastatin, benzyloxycarbonyl-Pro-Phe-CHO, FK-448, chicken ovoinhibitor, duck ovomucoid.
elastase	calcium	Elastatinal, methoxysuccinyl-Ala-Ala-Pro-Val-chloromethyl ketone (MPCMK), Bowman-Birk inhibitor, soyabean trypsin inhibitor, chicken ovoinhibitor, diisopropyl fluorophosphate, phenylmethylsulfonyl fluoride
carboxypeptidase A	zinc	EDTA, chitosan−EDTA conjugates, polyacrylate derivatives
carboxypeptidase B	zinc	EDTA, chitosan−EDTA conjugates, polyacrylate derivatives
		Membrane-Bound Proteases
aminopeptidase N	zinc, cobalt	amino acids, di- and tripeptides, EDTA, amastatin, bestatin, puromycin, bacitracin, phosphinic acid dipeptide analogues, α-aminoboronic acid derivatives, chitosan-EDTA conjugates, Na-glycocholate
aminopeptidase A	zinc, calcium	phosphinic acid dipeptide analogues, α-aminoboronic acid derivatives, puromycin, EDTA, 1,10-phenanthroline, epiamastatin
aminopeptidase P	zinc, mangan	bestatin, phophinic acid dipeptide analogues, α-aminoboronic acid derivatives, apstatin, epibestatin
aminopeptidase W	zinc	phosphinic acid dipeptide analogues, α-aminoboronic acid derivatives
leucin aminopeptidase	zinc, mangan, magnesium,	Bestatin, amastatin, phosphinic acid dipeptide analogues, flavonoid inhibitors, α-aminoboronic acid derivatives
dipeptidyl peptidase IV	zinc	*N*-peptidyl-*O*-acylhydroxylamines boronic acid analogues of proline and alanine
γ-glutamyl transpeptidase	magnesium	Acivicin (amino-(3-chloro-4,5-dihydro-isoxazol-5-yl)-acetic acid), L-serine borate
peptidyl dipeptidase A	zinc	ACE inhibitors (in theory)
carboxypeptidase M	zinc	D,L-2-mercaptomethyl-3-guanidinoethylthiopropanoic acid
carboxypeptidase P	zinc, mangan	Enterostatin, EDTA
neutral endopeptidase	zinc	1,10-phenanthroline, thiorphan ((2-mercaptomethyl-3-phenyl-propionylamino)-acetic acid), phosphoramidon, SQ 28,603 (*N*-[2-(mercaptomethyl)−1-oxo-3-phenylpropyl]-*β*-alanine)

the stability of the therapeutic agent in the intestine. On the other hand, the quantity of co-administered inhibitor(s) is essential for the intestinal stability of a peptide or protein drug. Various inhibitors of pancreatic and brush-border membrane-bound proteases are listed in Table 5.

An improved stability and absorption enhancement effect of these enzyme inhibitors on PP drugs has been demonstrated in different studies.[15,31,181−185] In a recent study, Sjostrom et al. reported the increased bioavailability of a proteolytic stable peptide, inogatran, during co-administration with the trypsin inhibitor.[186] Low bioavailability of inogatran was attributed to its binding with intestinal trypsin and trypsin-like enzymes in intestine and showed that the increased bioavailability effect in the presence of trypsin inhibitor was due to competitive displacement of inogatran from trypsin. Another promising strategy to overcome the enzymatic barrier by using enzyme inhibitors is to use auxiliary agents such as mucoadhesive polymers along with enzyme inhibitors. These polymers can provide intimate contact with GI mucosa, thereby excluding the presystemic metabolism of therapeutic polypeptide on its sojourn from delivery system to absorption membrane, and at the same time conjugated/co-administered enzyme inhibitors are able to inactivate proteases locally and specifically, which penetrate into the polymeric carrier system. Similarly, synergistic effects of absorption enhancers and protease inhibitors have also been studied. Ziv et al. reported use of a combination of an enhancer (sodium cholate) and a protease inhibitor (aprotinin) to achieve a 10% increase in amounts of insulin absorbed.[94,96,187]

Another approach to limit intestinal enzymatic activity is to transiently modulate the pH of intestinal contents to the pH minima for proteolytic enzyme activity. Lee and Sinko[188] used citric acid containing enteric-coated formulations of sCT to study the influence of intestinal pH change on sCT absorption in conscious normal beagle dogs. It was found that use of citric acid in the formulations resulted in lowering of intestinal pH and peak plasma concentrations of sCT always coincided with the pH decline. The increase in citric acid content in the formulations resulted in higher plasma sCT concentrations and plasma area under the curve values. The enhanced absorption effect was related to lower enzymatic activity (serine protease trypsin) and consequently better stability of drug in lowered intestinal pH. Sinko[188] suggested that this approach is more effective if release of drug and pH modifying additives is programmed to begin in the lower segment of intestine, where the spreading and dilution effects are minimal to give more significant and steady pH drop. However, this particular approach can be adopted only for PP drugs (e.g., sCT) that show extensive enzymatic degradation in the gut lumen but negligible hepatic first-pass elimination.

1. Mucoadhesive Polymers for Bioavailability Enhancement of PPs

Polyacrylate derivatives, such as poly(acrylic acid) and polycarbophil (mucoadhesive polymers), have been shown to enhance the membrane permeability of a number of PP drugs such as buserelin and insulin.[114,189,190] Several mechanisms for their permeability enhancing effects on PPs have been proposed.

(i) Mucoadhesive properties of these polymers enhance the contact between formulation and mucosal surface and thereby increase the residence time at the site of drug absorption. At the same time, the distance between released therapeutic polypeptide from the dosage form and the absorptive tissue is decreased.[191,192] The later effect results in reduced drug metabolism by luminally secreted proteases.

(ii) Ca^{2+} binding properties of these agents result in reduced extracellular Ca^{2+} and hence hampered integrity of TJ locally,[193] which consequently results in a penetration enhancing effect. However, it has been shown that Ca^{2+} ion chelation on either the basolateral side or basolateral and apical sides of cultured cell lines improves the epithelial permeability, whereas similar treatment on the apical side alone does not cause any improvement in permeability characteristics of the cell membranes.[194]

(iii) Inhibition of proteolytic enzymes present in GIT.[195] They bind to the essential enzyme cofactors calcium and zinc in the enzyme system, causing a conformational change resulting in enzyme autolysis and loss of enzyme activity.[196]

(iv) Polyacrylic polymers release numerous protons in intestine, creating a local temporary acidic environment in which luminal and pancreatic enzymes have low or negligible activities and thereby preventing PP drugs degradation by luminal enzymes. Bai et al. showed that degradation of insulin, calcitonin, and insulin-like growth factor I (IGF−I) was prevented by Carbopol polymers (934P, 974P, and 971P) in an in vitro system with saline as the incubation medium.[114] Inhibitory effect was correlated to the reduction in pH of the incubation medium by these polymers and proved that inhibition and change in pH was negligible or absent when saline was replaced with Tris buffer as the incubation medium.

(v) Direct interaction of polymers with enzymes reduces the free enzyme concentration and in part denatures the enzymes, thereby reducing the proteolytic activity of luminal enzymes.[197]

Hence, rather than being just 'adhesives', mucoadhesive polymers may be considered as multifunctional macromolecules with a number of desirable properties for their use as PP drug delivery adjuvants. Chitosan, a mucoadhesive poly(aminoglucan), and its derivatives have gained importance as excipients for *peroral* PP delivery systems. In a review, Bernkop-Schnurch gave a comparative description of the preparation, properties, and use of chitosan and its chemically modified derivatives in PP drug delivery.[198] Absorption enhancing effects of chitosan are ascribed to its paracellular absorption enhancing activity,[192,199] enzyme inhibitory capability,[200,201] and mucoadhesive properties.[202] Schipper et al. explained that interaction of chitosan with cell membrane due to positive charges on the polymer might result in a structural reorganization of TJ-associated proteins, giving it its paracellular absorption enhancement activity.[203] Chitosan per se is a poor inhibitor of peptidases[204] unless chemically modified by conjugating with enzyme inhibitors.[200,205] The mucoadhesive properties of chitosan are almost as strong as poly(acrylic acid).[202] It has been shown to produce comparable improvement of dDAVP intestinal absorption in the in vitro rat model to that produced by polycarbophil at the same weight concentration.[206]

Whether the protective and absorption enhancing effects of these polymers are sufficient to result in improved bioavailability of polymer-embedded PP drugs will mainly depend on the type of polymer and dosage form used. However, it is very likely that simple formulations with polyacrylate derivatives will not be able to exert a sufficient protective effect.[207] Hence, attempts have been made to improve the inhibitory activity of mucoadhesive polymers by covalent attachment of enzyme inhibitors such that the mucoadhesive properties of polymer and enzyme inhibitory properties of inhibitor are not lost. Such conjugates offer the advantages of reduced presystemic metabolism of PP drug, exclusion of undesired disturbance in digestion of nutritive proteins due to reduced dilution effects of inhibitors, exclusion of systemic toxic side effects of inhibitors, and site-specific targeting in GIT, and in all it may result in reduced dose requirements for enzyme inhibitors.[208] There are a number of reports using mucoadhesive polymer-inhibitor conjugates for PP drug delivery (Table 6); however, their applicability is limited at present due to extensive costs of certain enzyme inhibitors. Hence, there are attempts to synthesize less expensive enzyme inhibitor analogues and link them to bioadhesive polymers so as to guarantee low production costs in large-scale preparation of such conjugates that can be used as vehicles for *peroral* administration of PPs.[209,210]

D. Dosage Form Modifications

Another very important drug delivery approach for enhanced bioavailability of PP drugs involves design of suitable drug delivery systems that can obviate the enzyme and absorption barriers. Largely, such dosage forms rely on their ability to protect the drug from degradative GI enzymatic attack and deliver the drug at the most favorable site for drug absorption such as the large intestine where proteolytic activity is minimal. While developing such dosage forms, the concept of enzyme inhibitors, absorption enhancers, and/or bioadhesive polymers may also be coupled together so as to increase the efficiency of dosage form to deliver drugs to absorptive mucosa in its intact form and then subsequently increase the drug absorption into systemic circulation. Sinko et al. described successful improvement in sCT bioavailability in the intestine and vascular access port dog model by using two *peroral* formulations.[211] The formulations contained citric acid (to cause transient reduction in local pH) and lauroyl carnitine chloride or sodium taurodeoxycholate (permeation enhancers), thereby making use of two mechanisms via a reduc-

Table 6. Mucoadhesive Polymer-inhibitor Conjugates[a]

polymer−inhibitor conjugate	inhibitory activity toward	ref
carboxymethylcellulose−pepstatin	pepstatin	323
poly(acrylic acid)−BBI	chymotrypsin	207
poly(acrylic acid)−chymostatin	chymotrypsin	324
poly(acrylic acid)−elastatinal	elastase	325
carboxymethylcellulose−elastatinal	elastase	325
polycarbophil−elastatinal	elastase	325
chitosan−antipain	trypsin	326
poly(acrylic acid)−bacitracin	aminopeptidase N	327
chitosan−EDTA	aminopeptidase N, carboxypeptidase A	205,328
chitosan−EDTA−antipain, −chymostatin, and −elastatinal	trypsin, chymotrypsin, elastase, carboxypeptidase A, carboxypeptidase B, aminopeptidase N	201
chitosan−EDTA−BBI	trypsin, chymotrypsin, carboxypeptidase A, aminopeptidase N	198,329,330
chitosan−DTPA	carboxypeptidase A, aminopeptidase N	200
Na−CMC−BBI	trypsin, chymotrypsin, elastase	331
polycarbophil−pepstatin analogue	pepsin	332
Na−CMC−pepstatin analogue		

[a] EDTA: ethykenediamine tetraacetic acid. DTPA: diethylenetriamine pentaacetic acid. BBI: Bowman-Birk inhibitor. Na−CMC: sodium carboxymethylcellulose

tion in the proteolytic degradation in GI lumen by a pH lowering mechanism and a permeation enhancement. Some of the drug delivery systems that have been used with success for delivery of PPs are discussed in the following sections.

1. Matrix Carrier Systems: Nanoparticles, Microparticles, and Tablets

The use of colloidal drug carriers such as polymeric nano- and microspheres to achieve site-specific and improved drug delivery has found interest in the last 15 years. It has been shown that particles in the submicrometer range (up to 5 μm) can cross the intestinal wall intact:[212−215] (i) through M-cells of the Peyer's patches of the gut-associated lymphoid tissue (GALT); (ii) through normal epithelial cells (enterocytes); and (iii) by paracellular means. Mathiowitz et al. encapsulated insulin in a blend of poly(fumaric anhydride) and poly(lactide-*co*-glycolide) (PLGA) using a phase inversion nanoencapsulation method and reported an increased biological activity of insulin in fasting rats upon *peroral* administration.[215] The authors suggested uptake of microspheres into circulation by the cell lining of GIT as a possible reason for increased insulin activity. Similarly, an increased biological effect of insulin from insulin-loaded isobutyl 2-cyanoacrylate nanocapsules was shown to be due to combination of (i) protection of insulin against proteolytic degradation in the gut, (ii) passage of nanocapsules through intestinal mucosa into lymph ducts, and (iii) change in insulin distribution in the organism.[216] The penetrability of these multiparticulate systems to aqueous fluids, however, is a serious concern as it can render them susceptible to problems such as initial burst release and loss of protein protection. Additionally, the drug release from permeable polymeric particles involves diffusion and bulk degradation processes[217] and can be a cause for unpredictable drug release due to changes in the polymer matrix and matrix permeability to the drug. Lambert et al. described the preparation of surface erosion-controlled bioerodible poly(ethylene carbonate) microspheres containing G-CSF, which allowed very low in vitro initial drug release.[218] The advantage of using surface-eroding polymers such as poly(ortho esters) and poly(ethylene carbonates) is that the matrix is impermeable to body fluids and drug release is strictly controlled by surface erosion rather than diffusion through the matrix, making the drug unavailable to body fluids before it is released.

Most of the methods used for the preparation of multiparticulate delivery systems (microparticles and nanoparticles) are based on an emulsion (simple or multiple)−solvent evaporation or −solvent extraction scheme.[219−222] However, the common drawbacks of these methods are low encapsulation efficiency and reduced bioactivity of PPs after incorporation into microparticles.[221,223,224] Use of organic solvents (solvent for dissolving polymer, e.g., methylene chloride for PLGA) as the oil phase of an emulsion denatures PP drugs during the preparation. At the same time PP drugs, owing to their hydrophilic nature, tend to escape from the primary aqueous phase of multiple emulsions to continuous aqueous medium during a second emulsification step. Hence, the issues of large-scale production and achieving high drug loadings for highly water-soluble PP drugs in nano- and microparticles have remained a matter of concern. Attempts are being made to improve the encapsulation efficiency and bioactivity of encapsulated proteins. Recently discussed approaches include modifications in existing emulsion−solvent evaporation-based methods of microparticle preparation[225] (replacement of commonly used methylene chloride with lesser toxic organic solvents and addition of gelling polymers), use of additives[225] (such as trehalose and sucrose), development of all-aqueous encapsulation methods,[226] and hydrophobic ion pairing of PP drugs.[227−231] Ionic interaction of charged groups in proteins with the oppositely charged headgroup of amphiphilic molecules (phospholipids and fatty acid salts, e.g., sodium oleate) results in formation of a hydrophobic ion-pair complex with higher solubility in the organic phase of the emulsion system used for preparation of microparticles. The increased solubility of the ion-pair complex in the organic solvent

phase of the emulsion helps to improve the loading and conformational stability of PP drugs during the preparation of microparticles.

Though this unusual approach sounds promising, the uptake of microparticles into general circulation from the GI lumen has so far been very small (6−7% of 50 μm particles).[213] Presently, use of this approach is limited to *peroral* delivery of antigens for mucosal vaccines, where uptake of antigen carrying microspheres by Payer's patches of GALT stimulate production of immunoglobulin A in all mucosal sites.[220,232] Its applicability for systemic delivery of PP therapeutics is yet to be fully proved.[233] Attempts are being made to amplify the intestinal uptake of particulate delivery systems. Very recently, Russell-Jones and co-workers (Biotech Australia) proposed a nanoparticulate system surface modified by vitamin B_{12} (vitamin B_{12} derivatization of carboxylic groups on the surface of nanoparticles) for their vitamin B_{12}-mediated uptake upon *peroral* administration.[234] The authors reported that on reduction in the surface, substitution of nanoparticles with vitamin B_{12} resulted in reduced levels of their uptake by Caco-2 cells. Such nanoparticles have been reported to be capable of protecting the loaded PP drugs from degradation in the intestine and also transporting them into the circulation.[162] Similarly, uptake and transport of nanoparticles surface modified with lectins have been described for *peroral* antigen delivery.[166] Lectins are known to recognize and bind to specific carbohydrate and sugar residues on epithelial cell surface[167] and thus work as co-transporter in uptake and transcytosis of nanoparticles across intestinal cells.

Hillery et al.[235,236] combined two different approaches (i.e., use of particulate carrier and use of drug polymer conjugate) by synthesizing a novel drug polymer conjugate that formed its own nanoparticulate delivery system, the copolymerized peptide particle system (CPP). They described that an LHRH copolymeric compound (prepared using *n*-butylcyanoacrylate and peptide vinyl acetate as comonomers), when administered *perorally* to rats as about 100 nm particles, produced significantly higher activity as compared to a solution.[235−237] In another report, Larionova and co-workers[238] described preparation of aprotinin-loaded starch/bovine serum albumin mixed walled microcapsules using interfacial crosslinking method. The protein release was dependent on the enzymatic degradation of the microcapsules by α-amylase, and at the same time, it was protected from proteolytic enzymatic degradation due to enzyme inhibition activity of aprotinin. Kawashima et al. described the preparation of elcatonin-containing chitosan coated PLGA nanospheres using emulsion solvent diffusion method.[239] Intragastric administration of these mucoadhesive nanospheres to fasted wistar rats caused significant reduction in blood calcium levels for 48 h. Similarly, Kimura et al.[240] described about 2% bioavailability of insulin with significant and prolonged reduction of blood glucose levels from intragastric administration (in streptozotocin induced diabetic rats) of poly(vinyl alcohol)−gel spheres containing insulin and a protease inhibitor, thereby combining the effect of prolonged residence time of gel spheres in lower intestine with enzyme inhibition.

A matrix-based minipellet system prepared from collagen has been described for sustained *peroral* delivery of protein drugs.[241,242] Kim et al.[243] and Serres et al.[244] fabricated a temperature- and pH-sensitive polymeric bead system from linear terpolymers (poly(*N*-isopropylacrylamide-*co*-butyl methacrylate-*co*-acrylic acid) for the delivery of insulin and human calcitonin. It was found that in vivo biological activities of both the hormones in polymeric beads were improved and protected from gastric degradation. Another interesting strategy used by Bernkop-Schnurch and Gilge[245] involved preparation of tablets containing neutralized carbomer (sodium salt of Carbopol 934P), which was found to be capable of protecting embedded pepsin-degradable model protein (horseradish peroxidase) from pepsinic degradation in simulated gastric fluid. The authors explained that high buffer capacity of neutralized polymer helps to maintain the pH value of the delivery system above 5.5, and development of such a delivery system would ensure protection to embedded PPs therapeutic from pepsin that might diffuse into the delivery system upon its rapid swelling. However, high adsorptive binding properties of the neutralized Carbopol resulted in incomplete peroxidase release.

2. Self-Assembling Molecular Superstructures: Proteinoids

Micelles and vesicles are examples of the phenomenon of intermolecular association between similar structures. These structures are held together by the weak hydrophobic−hydrophilic interactions between the head and tail groups of the molecules; however, they exist only in solution and collapse in dry conditions. Self-assembled molecular superstructures, which can maintain their integrity on drying, have been reported for thermally condensed amino acid mixtures.[246] These amino acid mixtures have the ability to self-aggregate into sphere-like microparticles (0.1−10 μm size depending on amino acid composition) called "proteinoids".[247] These proteinoids are formed under acidic conditions from synthetic polymers of amino acids and get redissolved under conditions of high pH. They can hold a cargo of drug, including PPs and antigens. When administered *perorally*, proteinoids remain intact in the stomach, but on their discharge into the small intestine, they undergo spontaneous dissociation to release the drug, thereby helping the encapsulated peptide bypass proteolytic destruction in the stomach.[248] The proteinoids have been found to deliver therapeutically significant amounts of heparin and sCT, providing further support for the therapeutic possibility for *peroral* delivery of to-date *perorally*-inactive peptides.[246,249] A self-assembling lipid system for oral delivery of PP drugs is under development at Biotech Australia. The technique involves use of natural lipids to coat PPs so as to provide them protection from proteolysis and at the same time achieve their co-transport along with lipids during normal lipid uptake.

3. Vesicular Systems: Liposomes and Niosomes

The vesicular systems have shown great potential in *peroral* delivery of PP drugs in the recent past.[250−254] Their biodegradable and nontoxic nature (due to similarity of construction materials to integral components of biomembrane) and capability to encapsulate both hydrophobic and hydrophilic drugs makes them ideal drug carrier systems. However, a major drawback in using vesicular systems for *peroral* application of PP drugs is their low chemical and physical stability. The vesicular structures get easily degraded or disrupted by bile salts in GIT, exposing the incorporated PP drug to a harsh GI environment, and attempts are being made to overcome these difficulties. Arien et al. designed calcitonin-loaded liposomes that were stable in acidic medium.[255] Surface coating of liposomes can help to overcome their instability problem in acidic and bile salt containing GI fluids. Iwanaga and co-workers[256] showed that insulin liposomes coated with PEG or sugar chain portion of mucin can provide complete protection to insulin from enzymatic degradation in intestinal fluid. The coated liposomes were found to remain stable in acidic solution (pH 2.0) and in the presence of bile salts (sodium glycocholate, sodium taurocholate). Similar results of improved stability of *O*-palmitoylpullulan-coated liposomes in sodium cholate were earlier reported by Shegal and Rogers,[257] though for a non-protein/peptide drug cytosine arabinoside. It has been suggested that surface coating with PEG or mucin results in formation of a thick water layer on the liposome surface, which can effectively prevent the direct interaction of bile salts with lipid membrane.[256,258] As a consequence, oral administration of these insulin-loaded coated liposomes resulted in significantly decreased glucose levels as compared to those attained by insulin solution or uncoated liposomes. At the same time, the GI transit rate of liposomes was also found to be affected by surface coating and has been suggested as one of the reasons for altered bioavailability of encapsulated insulin from coated liposomes.[259] Iwanaga and co-workers[259] revealed that mucin-coated and PEG-coated liposomes were preferentially retained in different regions of GIT. In comparison to uncoated and mucin-coated liposomes, PEG−liposomes were found to spread widely in the intestinal tract with the highest values of mean transit time reflecting the lowest transit rate. Longer retention of mucin−liposomes in the stomach and PEG−liposomes in the lower region of the intestine were explained to be caused by interaction of mucin on the surface of the stomach with that of the liposomes and interaction of PEG with the intestinal surface, respectively.

Despite various difficulties associated with vesicular drug delivery systems, their inherent advantages have prompted researchers to use them for effective *peroral* drug delivery. On one hand, vesicles might be transported across the mucosal tissue,[260−262] while at the same time, the surfactants or lipids from which the vesicles are prepared might act as penetration enhancers and increase the flux of peptide drugs across mucosal tissue. Thus, use of lipid vesicular (liposomes) and nonionic surfactant vesicular (niosomes) systems have paved the way to circumvent membrane barriers and thereby promote the uptake of this 'difficult' class of drugs. Oral administration of chitosan-coated bioadhesive liposomes containing insulin to normal rats has been shown to result in marked reduction in basal blood glucose levels in comparison to uncoated liposomes or insulin solution.[263] The reduced basal blood glucose levels were maintained up to at least 12 h after administration of chitosan-coated liposomes, which was attributed to their bioadhesive properties, and it was presumed that insulin molecules released from liposomes in the mucus layer could be absorbed without being enzymatically degraded. These vesicles may also directly protect the drug against the enzymatic degradation in intestinal lumen.[264] At high concentrations (concentrations higher than critical micelle concentration) bile salts have been shown to protect insulin monomers from being exposed to enzymatic activities and degradation.[114] Yoshida et al.[265] prepared 9-desglycinamide 8-arginine vasopressin (dGAVP) loaded niosomes using stable surfactants (polyoxyethylene alkyl ethers) and reported increased stability and increased in vitro absorption of dGAVP across rat jejunum from niosomal formulation.

4. Liquid Emulsions

Liquid emulsions are another category of delivery systems that have been tried to deliver PP drugs via *peroral* route. Emulsion-based systems enhance drug absorption to systemic circulation in two ways:[266,267] absorption into lymphatic vessels via thoracic duct or absorption into hepatic circulation via mesentric veins. They also protect the drug from chemical and enzymatic breakdown in the intestinal lumen. The drug absorption enhancement activity from such dispersed systems is dependent on the type of emulsifying agent, particle size of dispersed phase, pH, solubility of drug, type of lipid phase used, etc. Water-in-oil microemulsions have been shown to enhance *peroral* bioavailability of PPs.[268,269] Formulation of *N*-acetylglucosaminyl-*N*-acetylmuramyl dipeptide in a water-in-oil microemulsion and its intraduodenal administration to rats successfully increased the bioavailability of muramyl dipeptide analogue up to 10-fold compared with simple solution.[270] The lipid phase of microemulsion was a medium chain fatty acid triglyceride, known absorption enhancing agents,[134] and was proposed to have caused an increase in the permeability of the intestinal wall for the peptide.

5. Colonic Drug Delivery Systems

On *peroral* administration the drug is exposed to a hostile acidic environment of the stomach and high concentrations of proteolytic enzymes in stomach and proximal regions of small intestine. Exposure to these conditions results in rapid inactivation of PP drugs.[271−273] In an attempt to protect insulin from harsh conditions of the stomach, Lowman and co-workers[274] prepared microparticles of pH-sensitive poly(methacrylic acid-*g*-ethylene glycol) cross-linked copolymers. This delivery system protected the loaded

insulin in the acidic environment of stomach; however, the drug was released from polymeric dosage form in the small intestine due to alkaline pH-induced swelling of polymer. The corresponding serum glucose lowering activity of microspheres on *peroral* administration to normal and diabetic rats confirmed the effectiveness of the delivery system, signifying the importance of PP drug protection in acidic conditions of the stomach. The protection of PP drugs in only the stomach is usually not good enough to ensure absorption, and it is important to avoid proteolytic degradation in the intestine also. The regional distribution of intestinal peptidases is not uniform throughout the length of the GIT and colon is measured as a site low in host enzyme activity.[58,275,276] At the same time, weekly alkaline conditions in the colon (as compared to acidic pH of stomach) are considered to be more hospitable to PPs from a stability point of view. Therefore, the colon offers an alternative absorption site for PP drugs following *peroral* administration.[277] Protecting or camouflaging the drug long enough to permit its release and uptake in the colon can provide an efficient means of colonic drug targeting. Broadly, design of a colonic drug delivery system can be based upon a site-specific chemical signal, i.e., pH difference between the small and large intestine, site-specific physical signal, i.e., presence of microbial enzymes (bacterial glycosidases, azoreductases) in the large intestine, time- and signal-dependent controlled drug release systems in which the drug release profile corresponds to transit times through a certain part of the GIT.

The systems designed on a pH differential approach most commonly employ methacrylic acid−methyl methacrylate copolymers (Eudragit), cellulose derivatives (cellulose acetate phthalate, hydroxypropyl methyl cellulose phthalate etc.) as coating polymers for tablets, pellets, and capsules. Alternatively, biodegradable azopolymers and saccharidic polymers that degrade as a result of enzymatic cleavage in the large intestine, such as copolymers of styrene and hydroxyethyl methacrylate cross-linked with a divinylazobenzene derivative, can also be used.[278] Saffran et al. reported a sustained pharmacological response of insulin and lysine-vasopressin in animals on *peroral* administration of solid dosage forms coated with azopolymers.[279,280] Cheng et al.[281] also reported similar results from azopolymer-coated insulin capsules. Pectin, a heterogeneous polysaccharide, has been found to be of use in providing protection to drugs during transit to the colon and thereby achieve colonic drug delivery.[282,283] Combinations of calcium salts and pectin have been used to prepare matrix tablets for colonic drug delivery as calcium pectinate remains insoluble and is not degraded by gastric or intestinal enzymes but gets degraded by colonic pectinolytic enzymes.[283] Sriamornsak[284,285] studied the suitability of pectin, in the form of calcium pectinate gel (CPG) beads, for colonic delivery of a model protein bovine serum albumin (BSA). It was shown that BSA could be protected from conditions during mouth to colon transit by controlling various process parameters of making CPG beads. Similarly, Mumper et al. described calcium alginate beads for *peroral* delivery of transforming growth factor-β_1.[286] Yeh et al. described the synthesis of pH-sensitive hydrogels with enzymatically degradable azoaromatic cross-links for colon-specific PP drug delivery.[287] In the low pH of the stomach and small intestine these hydrogels resist swelling, thereby protecting the drug; however, rapid and extensive swelling in the alkaline pH of the colon exposes the azoaromatic cross-links to azoreductase activity, resulting in drug release through gel degradation. The gel degradation mechanism can be controlled to be either a surface erosion or bulk degradation-like process by means of controlling synthesis reaction conditions and consequent network structure of hydrogels. Recently, Ramkissoon-Ganorkar et al. developed pH/temperature-sensitive polymers (terpolymers of *N*-isopropylacrylamide, butyl methacrylate, and acrylic acid) based insulin releasing beads and showed that different molecular weight polymeric beads can be used to target different regions of GIT.[288]

All the dosage forms described above are largely coating (e.g., enteric coating) and/or matrix (e.g., pH-sensitive hydrogel matrix) based, which depend on the pH and enzymatic activity of the colon for colon-specific drug delivery. The pH-dependent dosage forms are usually sufficient to delay the drug release but tend to release some part of incorporated drug during the transit through the intestine, e.g., pH-sensitive enteric coatings start disintegrating as dosage form reaches the alkaline conditions of small intestine. Enzymatic activity from colonic bacterial flora may show large inter- or intraindividual variations or a disease-dependent variability. These factors may result in variable and at certain times low drug bioavailability even from these dosage forms. Hence, time-controlled colon-specific drug delivery systems with a controllable but definite lag phase are promoted as alternative systems. These systems are based upon constancy in the small intestine transit times[289] and changes in pH in various parts of the intestine. Klokkers-Bethke and Fischer[290] developed a coating-based multiple unit delivery system capable of providing a lag phase long enough to give positioned drug release in the distal segment of GIT. To overcome the lack of lag phase from simple enteric-coated systems, they optimized a coating system comprising of multiple layers of enteric coating, acid coating, and water-insoluble coating. Two other colonic delivery systems are discussed in short.

a. Pulsincap System. This system is a nondisintegrating capsule consisting of a water-soluble enteric-coated cap and water-impermeable and -insoluble body. Drug formulation is filled and enclosed in the capsule body with the help of a water-swellable hydrogel plug. The enteric-coated cap dissolves on entering the small intestine, exposing the hydrogel plug to surrounding fluids. The plug starts swelling at a rate determined by its degree of cross-linking until it gets ejected from the body of the capsule at a predetermined time (Figure 3a).[291] Hence, hydrogel plug swelling acts as an additional means of controlling the lag phase after dissolution of enteric coating.

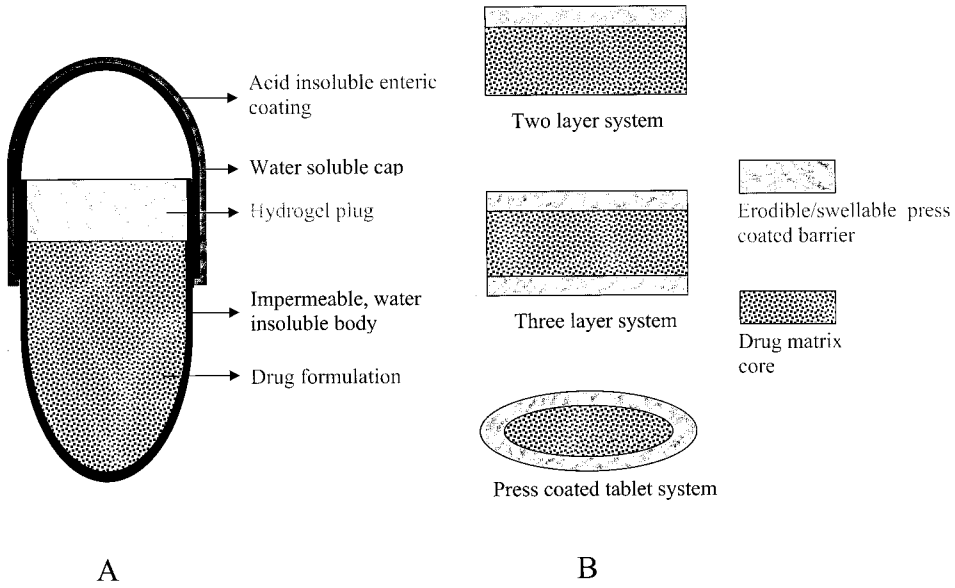


Figure 3. Colon drug delivery systems: Pulsincap (A) and Geomatrix (B) systems.

Such a delivery system can be effectively used for targeting the PP drugs to specific regions in intestine by modulating the lag phase.

b. Geomatrix System. A Geomatrix system is another time-release system designed to achieve a constant drug release with a predetermined and controllable lag phase. It is a multilayer tablet with a hydrophilic matrix core, containing the active ingredient, and one or more modulating barrier layers applied to the core by compression coating directly during the tableting process (Figure 3b).[292,293] The barrier layers can be of hydrophilic polymer (e.g., hydroxy propyl methylcellulose), enzymatically degradable hydrogels (e.g., pectin, calcium pectinate), or hydrophobic material (e.g., glyceryl monostearate, hydrogenated castor oil) the thickness of which can be optimized to achieve the desired initial lag phase. After the lag phase, rate and duration of drug release from the Geomatrix system is regulated by the exposed surface area and volume dimension of the core (coated−uncoated surface ratio).[292]

These drug delivery systems not only provide protection to the PP drugs in GIT area passed through during the lag phase, but also give an opportunity to preprogram the drug delivery rate to match the absorption capacity of the colon.

III. Future Directions

So far the problem of poor *peroral* bioavailability of PP therapeutics due to biological barriers of enzymes, mucus, and cell membrane has kept scientists busy, and the focus of their research efforts has largely remained on solving this problem using different approaches. It is now well understood that the stability of PP drugs in the presence of enzymatic degradation, absorption across the intestinal barrier, and mucocompatibility play an important role in determining their overall bioavailability.[294−297] However, the issues related to formulation development, where different drug delivery approaches are put in the shape of dosage form and drug product, and PP stability during the delivery system manufacturing process have remained unattended to a large extent. As an example, EDTA, bile acids, and surfactants that are known to be potential absorption enhancers can cause deaggregation of insulin and, hence, increase its rate of enzymatic degradation.[298−300] Similarly, Niu and Chiu[301] pointed out the possibility of PP degradation, e.g., during wet granulation process of tablets manufacture due to exposure to water and/or heat application for drying process. Various mechanical and chemical stresses that the drug undergoes during pharmaceutical operations in combination with inherent instabilities of PP drugs makes the job of the formulation scientist even more difficult and must be given consideration while devising strategies for PP drug delivery. Working in that direction, Balasubramanium et al.[302] studied liposomes as formulation excipients for protein pharmaceuticals. KP6β, an 81 amino acid yeast killer toxin secreted by fungal pathogen *Ustilago maydis*, was studied for its folding/unfolding properties. It was concluded that partially folded intermediate structures of the protein could interact with liposomes through protein intercalation into the bilayer membrane. This interaction stabilizes the intermediate structures against aggregation, preventing the loss of global fold and activity of proteins, thereby leading to increased physical stability of proteins. Similarly, irreversible inactivation (insoluble noncovalent aggregation and hydrolysis) of large globular proteins, such as bovine serum albumin, recombinant human basic fibroblast growth factor, and bone morphogenic protein, in PLGA systems due to acidic microclimate pH and intermediate water content existing in the polymer can be avoided by incorporation of basic additives in the formulation.[303] Co-encapsulation of magnesium hydroxide, a poorly water-soluble inorganic base, results in stabilization of bovine serum albumin in PLGA implants through neutralizing the acidic microclimate pH in the polymer.[304] However, a proper selection of base type, base loading, and protein loading are important to neutralize the acidic micro-

Table 7. Some of the Companies and Tecnologies that Are Involved in Development of Orally Active PP Formulations

company	technology	products currently available or under development
Altus, United States	nondegradable or biodegradable crystal matrix [cross-linked enzyme crystals (CLEC), cross-linked protein crystals (CLPC)] as delivery vehicles for proteins	calcitonin and other polypeptides
Alza Corporation, United States	biodegradable microparticle absorption via receptor-mediated endocytosis, combination of absorption enhancers	undisclosed
Amgen, United States	protein−vitamin B_{12} conjugates, pegylation, combination of absorption enhancers	vitamin B_{12}−interferon conjugate, PEG-G-CSF, PEG-INF-con
Biotech Australia, Australia	vitamin B_{12}-mediated delivery of PP and nanoparticles, self-assembling lipid systems, lipid emulsion systems	leutinizing hormone releasing hormone analogues, G-CSF, erythropiotin, interferons
Cortecs, United Kingdom	microemulsion technology	oral salmon calcitonin, oral insulin (macrulin)
DanBioSyst, United Kingdom West Company, United States	combination of absorption enhancers	undisclosed
Elan Corporation, Ireland	localized drug absorption system (LOCDAS), oral carrier-assisted drug absorption system (OCAS)	undisclosed
Eli Lilly, United States	oral drug delivery, solid oral dosage form[a]	oral insulin, recombinant parathyroid hormone (Forteo),[a] recombinant growth hormone (humatrope)[a]
Emisphere Technologies, United States	modified amino acid delivery agents, proteinoids	oral hGH, oral insulin, oral interferon
Flamel Technologies, France	polymeric delivery systems	trademarked systems Medusa (nanoparticle based drug carrier system) and Micropump (microencapsulation drug delivery system)
Genentech, United States	in collaboration with oral/depot delivery firms	long acting rhGH
Generex Biotech, Canada	oral delivery for large molecule drugs, aerosolized aqueous solution of drug containing mixed micelles of absorption enhancers for buccal drug delivery	oral insulin (Oralgen), Macrotonin for osteoporosis, Pseudostat for cystic fibrosis
MacroMed, United States	biodegradable polymeric drug carriers	undisclosed
Nobex, United States (formerly Protein Delivery, United States)	amphiphilic polymers and covalent conjugation	oral insulin (M2), oral enkephalin, oral calcitonin, oral parathyroid hormone
Novartis Pharma, Switzerland	oral solid dosage form[a]	salmon calcitonin[a]
Pharmaceutical Discovery, United States	microparticles	undisclosed
Pharmos, United States	bioadhesive microemulsion	undisclosed
Quadrant Healthcare, United Kingdom	oral delivery of macromolecules	undisclosed
Regeneron, United States	oral drug delivery[a]	Axokine[a]
Shire Laboratories, United States	build library of excipients that can increase absorption via specific pathway	Enkephalin-like peptide, leuprolide, leptin
Unigene, United States	manufacturing and novel drug delivery technology for oral delivery of amidated peptides, combination of absorption enhancers	oral calcitonin and several different peptides
VectorPharma, United States	solid lipid nanospheres	Calcitonin and other polypeptides

[a] In collaboration with Emisphere Technologies, United States.

climate throughout the polymer matrix. Hovgaard and co-workers[297] showed that use of dodecylmaltoside as a stabilizer in insulin preparation not only prevented protein's aggregation, but the stabilized insulin was found to be more resistant to intestinal enzymatic degradation and consequently result in increased absorption of insulin from stabilized complexes when administered intraduodenally. Hence, with these types of studies, currently there is an attempt to look at the *peroral* PP delivery from a different angle of PP product development. With the hopes of PP drug products surfacing, these studies have a drift in their objectives from being simply exploratory in nature (in terms of finding out the possibility of *peroral* delivery) to real-time formulation development and related problems. This is also reflected by the number of companies that are involved in developing *peroral* PP formulations (Table 7) and various strategies under development to meet the challenge.[305] The list is not complete, and there are many more corporations working in collaboration with their business partners to develop orally active PP drug products. The formulation development, scale-up, and production problems may vary for different PPs and drug delivery systems and for each combination of a PP with a particular delivery approach. This makes it prudent to conduct studies related to selection and optimization of a suitable delivery approach, formulation components, and processing conditions for a particular PP therapeutic on an individual product−by-product basis. The problems encountered therein would also need to be resolved on an individual basis.

IV. Closing Thoughts

Protein and peptide drugs are currently administered as parenteral therapies owing to their poor bioavailability from different nonparenteral routes of administration, including the *peroral* route. Among the various routes, *peroral* route has been most intensively investigated for PP drug delivery due to the advantages of patient compliance and acceptability associated with this route. The availability of safe and effective *peroral* PP drug products would avoid their repeated parenteral administrations. Various strategies that have been used in the past to achieve *peroral* delivery of PP drugs include prodrug design and use of enzyme inhibitors and absorption enhancers; however, success has remained very limited. Only recently the concerted efforts in various laboratories across the world have resulted in identification and development of newer strategies, which can be used for *peroral* PP drug delivery. These include the pegylation approach, targeting to carrier proteins in GIT, conjugation of PPs to various absorption enhancers, or enzyme inhibitors or mucoadhesive polymers. Use of delivery agents, developed by researchers at Emisphere Technologies Inc., to achieve increased *peroral* absorption of PP therapeutics is now established beyond any doubt. Although this approach is still under development and underlying mechanisms are not very clearly understood, it is one of the most attractive and promising options currently. In fact, it is the better understanding of absorption mechanisms and involvement of carrier proteins, auxiliary agents, GALT, P-glycoprotein, etc., in absorption processes that has helped researchers in taking up the challenge and be more systematic and rational while devising their strategies to achieve the target of acceptable *peroral* bioavailability for PP drugs. Various strategies to develop *peroral* PP delivery systems have been discussed in the present review, and an overview of the present scenario of the research efforts and the future in terms of the areas of attention and direction has been presented. Having designed, developed, and proved the potential of some of the approaches, now the efforts are on to using different available options in combination, thereby supplementing each other, with simultaneous attention on economically viable commercial-scale production of PP drug products so as to bridge the gap between a research concept and market product.

In conclusion, it can be said that challenges to develop *peroral* PP formulations are still significant and the quest to overcome them is still on. Potential advantages of breakthrough technology in this area justify continued efforts to identify and optimize various approaches for maximizing PP absorption. Chemical approaches of enhancing PP drug delivery (prodrug development, pegylation, small molecular complex-forming agents) have shown promise. Approaches based on modulation of GI absorption barrier properties (absorption enhancers, enzyme inhibitors, mucolytic agents, intestinal pH modulation) can be very useful provided apprehensions about their toxic manifestations are overcome. The possibilities are to optimize the agents and their concentration levels so as to localize the transient barrier modifying properties and keep it to minimally toxic levels. Drug delivery system-based approaches can be effectively combined with other strategies to provide maximum protection from degradation and achieve drug delivery at the preferred site of absorption. With the current pace of research and especially recent developments at hand, the idea of *peroral* PP drug delivery looks to be more convincing than ever before. Considering the number of PPs finding applications as therapeutic agents and the necessity-driven research efforts in the field of *peroral* drug delivery, it may be stated that not very far from now the GIT, the 'toughest' barrier, may be overcome. Success, in our opinion, will depend on how well different approaches can be utilized in harmony.

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VI. Note Added in Proof

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