Available online at www.sciencedirect.com
SCIENCE DIRECT®
International Journal of Pharmaceutics 294 (2005) 201–216
international journal of pharmaceutics
www.elsevier.com/locate/ijpharm

Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium

Nazila Salamat-Miller, Thomas P. Johnston *

Division of Pharmaceutical Sciences, Room 211A, School of Pharmacy, University of Missouri-Kansas City, Kansas City, MO 64110-2499, USA

Received 11 October 2004; received in revised form 12 January 2005; accepted 27 January 2005

Abstract

The intent of this paper is to update the reader on various strategies which have been utilized to increase the paracellular permeability of protein and polypeptide drugs across the intestinal epithelium. Structural features of protein and polypeptide drugs, together with the natural anatomical and physiological features of the gastrointestinal (GI) tract, have made oral delivery of this class of compounds extremely challenging. Interest in the paracellular route for the transport of therapeutic proteins and polypeptides following oral administration has recently intensified and continues to be explored. The assumption that molecules with a large molecular weight are not able to diffuse through the tight junctions of the intestinal membrane has been challenged by current research, along with an increased understanding of tight junction physiology.

Keywords: Paracellular permeability; Therapeutic polypeptides; Molecular geometry and size

1. Introduction

Obstacles associated with the absorption of therapeutic proteins and polypeptides following oral administration are intriguing challenges for pharmaceutical scientists. Oral administration of therapeutic polypeptides is notoriously difficult due to their high molecular weight, hydrophilicity, and susceptibility to enzymatic inactivation in the GI tract. The latter property, which is the natural function of the GI tract so that nutrients may be absorbed, has made the oral delivery of protein and polypeptide drugs extremely difficult.

An excellent review of the mechanisms for the paracellular intestinal absorption of protein and polypeptide drugs was provided by Nellans (1991). Pauletti et al. (1996a) reviewed the structural requirements for intestinal absorption of peptide drugs. Progress in the oral delivery of therapeutic proteins, such as insulin, calcitonin, human growth factor, and interferons, has recently been reviewed by Shah et al. (2002). One interesting feature of intestinal paracellular absorption of polypeptides is the role of the Na^+-glucose cotrans-

* Corresponding author. Tel.: +1 816 235 1624; fax: +1 816 235 5190.
E-mail address: johnstont@umkc.edu (T.P. Johnston).

0378-5173/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2005.01.022

porter on its regulation. This topic, which will also be addressed in this paper, has previously been reviewed by Turner (2000).

Tight junction physiology and its influence on the paracellular permeability of protein and polypeptide drugs is currently the subject of intense research. Examples of macromolecules that have been studied with regard to their paracellular transport across either the intestinal epithelium or a monolayer of Caco-2 cells include intact proteins (Gardner, 1984) and high-molecular-weight probes, such as polyethylene glycol (PEG) 4000, inulin (5500 Da), and dextran 4000 (Tanaka et al., 1995; Lane et al., 1996). Additionally, the paracellular diffusion of high-molecular-weight proteolytic enzymes across Caco-2 cell monolayers (Bock et al., 1998), the highly regulated passage of leukocytes through tight junctions (Burns et al., 2001), and the permeation of poly-D-glutamate (PDGlu) across both a Caco-2 monolayer and rat intestinal membrane (Salamat-Miller et al., 2005), are all examples which serve to support the premise that the intestinal epithelium is indeed permeable to large proteins and polypeptides. Therefore, it would appear that the name 'tight junction' is not entirely appropriate terminology, since, in some epithelia, tight junctions are rather 'leaky'. It is currently known that many endogenous compounds regularly diffuse through the paracellular pathway of the tight junctions (Anderson and Cereijido, 2001).

An update on the strategies used to increase the paracellular transport of protein and polypeptide drugs begins with a discussion of tight junctions in the intestinal epithelium. A correlation between the structure of the *zonula occludens* and the passive electrical permeability of several simple epithelia was demonstrated by Claude and Goodenough (1973). Claude (1978) developed a model that related transepithelial electrical resistance to the number of strands in the tight junction (Fig. 1). It should be mentioned that although this model replicated the characteristics of many tissues, some cell types, such as Type I and II MDCK cells, do not obey this hypothesis (Stevenson et al., 1988). Nevertheless, Claud's hypothesis has proved to model the behavior of many tissues.

The molecular nature of the proteins that line the tight junctional channels were ultimately identified as occludins (Furuse et al., 1993) and claudins (Furuse et al., 1998). The presence of different isoforms of the

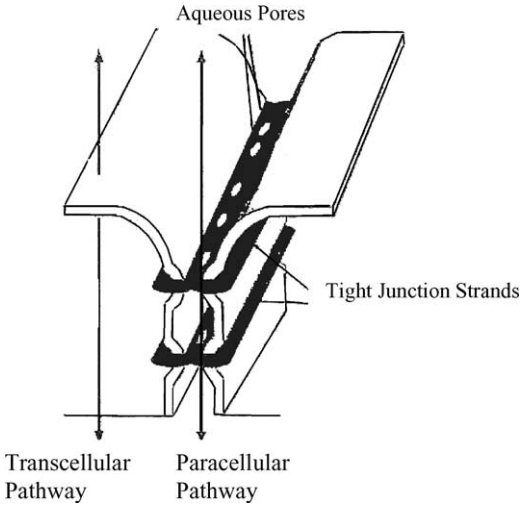

Fig. 1. Aqueous pores in the paracellular pathway (Reuss, 2001), with permission from CRC Press.

claudins (Cld-1, Cld-2, and Cld-3) in the tight junction strands result in the formation of heterogeneous pores with a range of diameters and charges (Reuss, 2001), although the tight junction is generally accepted as being negatively charged overall and therefore selective for positively charged permeants ('cation selective'). The presence of aqueous pores that can vary between an open and closed state was postulated by Claude (1978). Madara et al. (1986) also postulated the time-dependent opening and closing of tight junction pores during the paracellular transport of molecules. Thus, paracellular permeation of different compounds is based on the overall dimensions of the permeant and the particular population of tight junctions the permeant happens to encounter during its random diffusion down a concentration gradient. It should be mentioned that the tight junctions of Caco-2 cells are not affected by physiological processes such as villous tip 'sloughing off' of cells. Hence, there exists the potential for pore radii to be considerably different and paracellular permeation to vary along the crypt to villous axis; properties that cannot be accounted for using a Caco-2 cell monolayer model. For this reason, as well as the desire to incorporate mucus-secreting intestinal goblet cells, HT-29 cells, either alone or co-cultured with Caco-2 cells, have been used to study intestinal paracellular drug absorption (Collett et al., 1996; Walter et al., 1996).

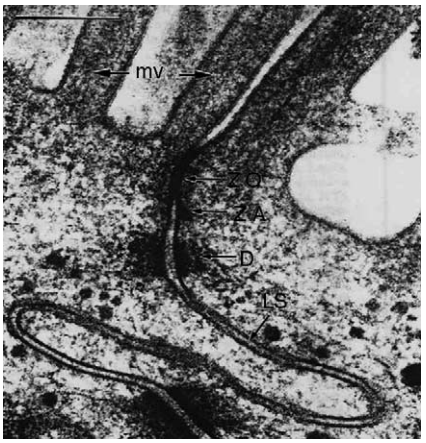

Fig. 2. The intercellular junctions between two human colon Caco-2 cells in culture: D, desmosomes; LS, lateral space; mv, microvilli; ZA, *zonula adherens*; ZO, *zonula occludens* or tight junction. Bar equals 200 nm. Reprinted from Adson et al., with permission from John Wiley & Sons.

The tight junction is the most apical portion of the junctional complex in the paracellular route. This thin network forms a continuous belt around the cell and seals the intercellular space (González-Mariscal et al., 2001). It has a depth of 100–800 nm (González-Mariscal et al., 2001). The functions of tight junctions can be summarized as (1) a fence-function, which maintains the apical/basolateral polarity in cell layers and inhibits the diffusion of lipid molecules from one cell to another, and (2) a gate-function that regulates the passage of ions and molecules through the paracellular pathway. In the tight junction region, the lateral membrane appears to fuse at certain points, informally known as 'kisses' (Fig. 1). The multiple layer of tight junctional strands presents a rate-limiting barrier for the free diffusion of hydrophilic molecules (Fig. 1). Fig. 2 shows the convoluted paracellular pathway distal to the *zonula occludens* or tight junction that a drug molecule must follow during paracellular transport.

2. The paracellular pathway of drug absorption

The paracellular route of drug transport has received much attention in recent years. Not only is it deficient in proteolytic activity, but it is also an aqueous-filled channel through which polypeptide drugs prefer to diffuse. The total area of the paracellular pathway, relative to the transcellular route, has been reported to range from 0.01% (Pappenheimer and Reiss, 1987) to 0.1% (Nellans, 1991). Considering that the intestinal epithelium has more than 2×10^6 cm^2 of surface area (Fasano, 1998a), the corresponding values of the paracellular surface area ranges from 200 to 2000 cm^2. This surface area should not be underestimated, since even the absorption of minute quantities (pM–nM range) of a protein or a polypeptide drug may be sufficient to exert their required biological effect.

As mentioned above, it has been shown that most protein and polypeptide drugs diffuse through the aqueous-filled tight junctional pathway due to their hydrophilic nature. General examples of paracellular permeability for this class of compounds have been cited by Kobayashi et al. (1995), He et al. (1996), Lang et al. (1997), and Pauletti et al. (1997). Some examples of peptide drugs that have been shown to permeate the intestinal mucosa via the paracellular route include octreotide (Drewe et al., 1993; Jaehde et al., 1994), potent analogs of vasopressin (Lundin and Artursson, 1990), thyrotropin-releasing hormone (TRH) (Thwaites et al., 1993), peptidomimetic renin inhibitors (Walter et al., 1995), and salmon calcitonin (Lee and Sinko, 2000).

New strategies to increase the paracellular permeability of protein and polypeptide drugs across the GI tract continue to be explored. Generally, these strategies may be classified into two major categories; namely, (1) physicochemical modification of the permeant, and (2) modulating the tight junctions associated with the paracellular pathway. Prior to a discussion of deliberately altering the physicochemical properties of a permeant to increase its paracellular transport, general physicochemical properties of the permeant, especially as it relates to polypeptides, will be considered.

3. Physicochemical properties of the permeant

A survey of the literature on parameters affecting the paracellular transport of proteins and polypeptides demonstrates the critical role of overall ionic charge and molecular weight for this route of transport (Adson et al., 1994; Horibe et al., 1997; Pauletti et al., 1997). The effect of molecular weight on the paracellular transport of various drug molecules has been demonstrated by many authors (Horibe et al., 1997; Matsukawa et al., 1997; Dodoo et al., 2000). In our

laboratory, we have focused on just one of the physicochemical properties associated with a polypeptide. We have investigated whether the secondary structure of various model homopolypeptides (synthesized using the same amino acid), which are incapable of forming tertiary structure, influences their paracellular transport. Normally, formation of tertiary structure involves many different amino acids that are capable of forming mostly non-covalent bonds, such as electrostatic interactions, hydrogen bonds, and hydrophobic interactions, as well as covalent bonds, such as disulfide bridges and isopeptide linkages.

Among the physicochemical properties of polypeptides, little or no emphasis has been placed on the overall molecular dimensions or the "shape" of the permeant, primarily because small molecules have typically been used in this type of research. However, the three-dimensional structure of larger polypeptides has traditionally been assumed to correlate with their molecular weight, without paying much attention to the particular shape of these molecules. For instance, spherical, prolate ellipsoid, oblate ellipsoid, elongated, extended rod-shaped, linear, coiled, and star-like are all examples of three-dimensional shapes that have been described for a variety of macromolecules. The flexibility or rigidity of a molecule is yet another factor that cannot be described by molecular weight alone.

Based on a review of the literature, peptides with no more than six amino acids have been utilized to conduct diffusion studies to address issues such as the effect of molecular weight, overall ionic charge, introduction of a β-turn, etc. on restricted versus unrestricted paracellular diffusion. Therefore, the paracellular diffusion of larger polypeptides across Caco-2 cell monolayers and the intestinal epithelium is still largely unexplored.

4. Effect of overall molecular flexibility on paracellular drug transport

The flexibility or rigidity of a polypeptide plays an important role in defining the overall geometry of the molecule. There have been no in-depth investigations of the effect of a polypeptide's overall molecular geometry/shape on its paracellular diffusion, both in vitro and in vivo. As mentioned above, our laboratory has investigated how the secondary structure of various model homopolypeptides, which are incapable of forming permanent tertiary structure, influenced their paracellular transport across both a Caco-2 cell monolayer and rat intestinal membrane in situ (Salamat-Miller et al., 2005). One investigation which did evaluate the effect of secondary structure of a small peptide on its transport across a Caco-2 monolayer demonstrated that introduction of a β-turn motif into the molecule resulted in an increase in lipophilicity (Knipp et al., 1997). With an increase in the peptide's lipophilicity, the primary route for its transport across the Caco-2 cell monolayer changed from paracellular to transcellular (Knipp et al., 1997).

It is well established that polypeptides exhibit different secondary structures depending on both their solution microenvironment and primary amino acid sequence. Hence, they are able to exhibit different secondary structures, such as a random coil (RC), α-helix, and β-sheets and β-turns. A change in secondary structure normally causes a polypeptide to adopt a different overall three-dimensional structure. Using a series of water-soluble probe molecules with different overall geometries, one study demonstrated a non-linear relationship between molecular weight and paracellular permeability (Hollander et al., 1988). These authors suggested that there was not necessarily a positive correlation between the probe's molecular weight and its overall molecular dimensions. Other authors have suggested that molecular volume of a polypeptide is a more important factor with regard to paracellular permeability than molecular weight (Hamilton et al., 1984). Using rat intestinal membrane, Lane et al. (1996) have demonstrated a positive correlation between the paracellular permeability of large hydrophilic probes and their molecular cross-sectional diameter. One interesting study reported that the permeability of various hydrophilic molecular markers across both rat intestinal membrane and Caco-2 cell monolayers was not inversely related to their molecular weight (Artursson et al., 1993). In other words, a molecule with a higher molecular weight (PEG 194–502 g/mol) demonstrated a 6- to 28-fold greater paracellular permeability than a compound such as mannitol (182 g/mol). These authors suggested that molecular flexibility of PEG could have played a role in this finding (Artursson et al., 1993). Based on the above investigations, it appears that parameters other than molecular weight may be more predictive of a molecule's potential to undergo paracellular transport.

The influence of overall molecular geometry on the diffusion behavior of polymers has also been demonstrated in the chemical engineering literature. The effect of overall molecular dimensions and geometry of synthetic polymers on their passage across glomerular capillary walls has been investigated using ficoll (a highly branched copolymer of sucrose and epichlorohydrin) and dextran (a flexible coiled polymer of 1,6-glucopyranose) as model polymers. These two polymers were selected to represent a rigid sphere and a deformable random coil, respectively (Bohrer et al., 1979). The greater permeation observed for dextran across both a porous synthetic membrane and the glomerular capillary wall was attributed to a decreased resistance towards diffusion due to molecular deformation and flexibility inherent in the RC structure (Bohrer et al., 1979, 1984).

Another recent investigation with polymeric drug carriers compared poly(amidoamine) (PAMAM) dendrimers to PEG molecules for their ability to cross microvascular endothelium and enter the interstitial tissue of a hamster cremaster muscle preparation (El-Sayed et al., 2001). Although the PAMAM dendrimers had comparable molecular weights to PEG, the authors concluded that the molecular geometry and charge of the PAMAM dendrimers resulted in a faster rate of transport across the endothelium (El-Sayed et al., 2001). While the studies mentioned above describe the effect of molecular dimensions on the permeation of synthetic or natural polymers across various biological membranes, there are no similar reports of the importance of overall molecular dimensions/geometry on the diffusion of extended polypeptide chains across biological membranes.

Our laboratory has recently demonstrated a positive correlation between the overall molecular flexibility associated with randomly coiled poly-D-glutamic acid and its enhanced paracellular transport relative to the α-helix conformer of the same polypeptide (Salamat-Miller et al., 2005). This study investigated hindered (restricted) paracellular diffusion of both secondary structures of poly-D-glutamic acid across Caco-2 cell monolayers and rat intestinal epithelium in situ (Salamat-Miller et al., 2005). Poly-D-glutamic acid provided an ideal model to evaluate how changes in the secondary structure of a polypeptide (with its associated changes in overall molecular dimensions or geometry) affected its rate and extent of paracellular transport/absorption.

In our studies mentioned above, 26.6 kDa poly-D-glutamic acid (PDGlu) was used as a model polypeptide that exhibited a random coil structure and which possessed an overall negative charge at neutral pH. This same molecule at pH 4.7 was used as a model polypeptide in which the majority (∼70%) of the residues adopt an α-helix secondary structure (Johnson and Tinoco, 1972). At pH 4.7, a smaller fraction of the carboxylic acid groups exist as the carboxylate anion (COO^-). Thus, PDGlu at pH 4.7 carries less negative charges overall than does PDGlu at pH 7.4. Fig. 3A depicts the cumulative amount of the randomly coiled conformer of PDGlu which diffused across a Caco-2 cell monolayer versus time (Salamat-Miller et al., 2005). No paracellular transport across the Caco-2 cell monolayer was observed for the α-helix conformer of PDGlu. Fig. 3B shows the plasma concentration of each secondary structure of PDGlu in the plasma of rats versus time following placement of an equimolar dose in an isolated segment of rat duodenum. The values of the apparent permeability (P_{app}) of PDGlu in the random coil conformation across a Caco-2 monolayer and rat intestinal membrane were $(7.0 \pm 1.2) \times 10^{-7}$ and $(4.8 \pm 0.2) \times 10^{-6}$ cm/s, respectively (Salamat-Miller et al., 2005).

Our experiments which investigated the paracellular transport/diffusion of two different secondary structures of the same polypeptide (PDGlu) demonstrated unequivocally that: (1) transport was by the paracellular pathway only (use of colchicine and sodium azide ruled out adsorptive endocytosis and active transport, respectively, and pretreatment of Caco-2 cells with EGTA resulted in a 3.3-fold increase in the amount of the polypeptide transported), (2) Caco-2 cell monolayer integrity was not compromised by a reduction in pH to 4.7 (simultaneous mannitol and diazepam transport studies, in which PDGlu was included in the donor solution, demonstrated no change in either the rate or extent of these two marker molecules of paracellular and transcellular transport, respectively), (3) no trace of either secondary structure of PDGlu was detectable in either the cytosol or cell membrane following the diffusion studies across a Caco-2 cell monolayer, (4) paracellular transport of PDGlu was secondary structure-dependent (the randomly coiled conformer of PDGlu demonstrated a significantly ($p < 0.01$) greater

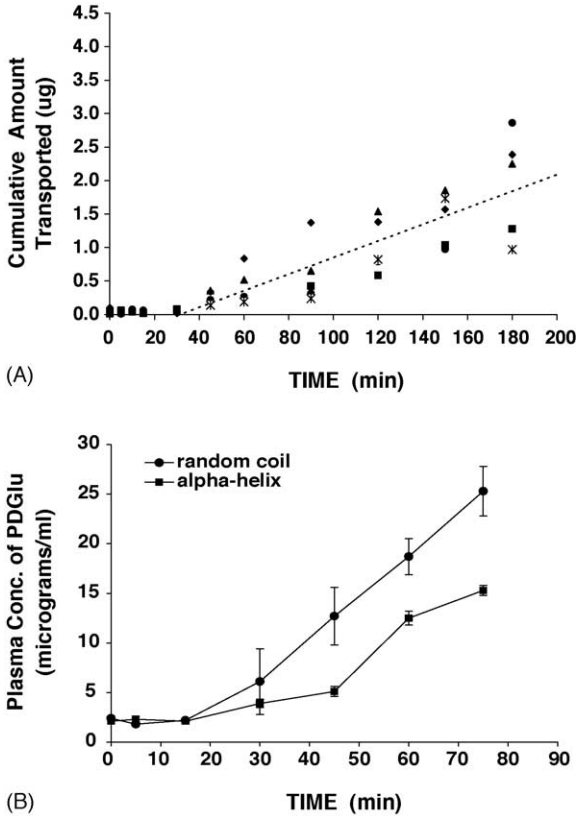

Fig. 3. (A) The cumulative amount of PDGlu in the RC conformation that was transported across Caco-2 monolayers. No paracellular transport was detected for the α-helix secondary structure of PDGlu. Symbols represent the values for five different experiments. The dashed line represents the 'best-fit' of the data from 30 min onward using linear regression ($r = 0.9865$; slope = 682 ng/h with an apparent lag-time of 30 min). (B) The plasma concentration-time profiles for the random coil (●) and α-helix (■) secondary structures of PDGlu following placement of an equimolar dose in a 6–8 cm length of rat duodenum. Symbols represent the mean value ± standard deviation.

value of P_{app} for paracellular transport when compared to the α-helix secondary structure of the same molecule), and (5) the rate and extent of paracellular transport of both secondary structures of PDGlu was much greater across rat intestinal membrane than through the tight junctions of a Caco-2 cell monolayer (Salamat-Miller et al., 2005). Interpretation of several of our key experimental findings is presented below.

With regard to secondary structure-dependent transport, both across a Caco-2 cell monolayer and rat intestinal membrane, the authors have suggested that the enhanced paracellular transport we observed with randomly coiled PDGlu may have resulted from molecular flexibility and subsequent deformation during its entrance and passage through the tight junction (Salamat-Miller et al., 2005). Indeed, the influence of structural flexibility of a polypeptide on its paracellular transport was previously predicted by Pauletti et al. (1996a) in which the authors stated, "*for peptide drugs possessing a high degree of conformational flexibility, it might be possible that even larger molecules can permeate the tight junctions*". Our findings, which evaluated the paracellular transport of each secondary structure of PDGlu across a Caco-2 cell monolayer and intact rat intestinal membrane, would tend to support the premise that structural flexibility, as it relates to a macromolecule's capacity to flex or deform during restricted diffusion, is more important than either the polypeptide's overall ionic charge, molecular weight, or relatively large hydrodynamic radius (R_H) (Salamat-Miller et al., 2005). The paracellular transport of a 26.6 kDa, negatively charged polypeptide through negatively charged tight junctions, without the aid of tight junction expanders, is proof of this concept.

In our experiments with PDGlu, we suggested that the observed differences in the rate and extent of paracellular transport for both secondary structures of PDGlu across a Caco-2 cell monolayer versus rat intestinal membrane may be explained by the "leaky" nature of tight junctions in rat duodenum (Salamat-Miller et al., 2005). TEER values associated with this tissue are classified as "leaky" (TEER values ranging from 30 to 100 $\Omega\,cm^2$) compared to Caco-2 cell monolayer tight junctions, which are categorized as "intermediate to tight" (TEER values ranging from 230 to 1000 $\Omega\,cm^2$) (Ward et al., 2000). An intact small intestine which is fully innervated, actively exchanging oxygen and carbon dioxide between the blood and adjacent tissue, has a significantly greater absorptive surface area, contains physiologically active tight junctions (tight junctions opening and closing over time), and has a smaller unstirred aqueous diffusion layer adjacent to the microvilli, are all factors which may have contributed to different values of P_{app} for paracellular transport across intestinal tissue compared to corresponding measurements obtained using Caco-2 cell monolayers (Lennernäs et al., 1997). Indeed, it has already been demonstrated that drug permeabil-

ities across Caco-2 cell monolayers are lower compared to the intestinal epithelium of various species and more similar to colon permeability (Stewart et al., 1995; Rubas et al., 1993; Artursson et al., 1993).

5. Effect of overall ionic charge on paracellular drug transport

Another important molecular property to consider with regard to paracellular transport of polypeptides is the polypeptide's overall ionic charge. The effect of charge on the passive paracellular diffusion of peptides across Caco-2 monolayers has been previously described (Pauletti et al., 1997). These authors reported that the paracellular diffusion of charged, small peptides across a Caco-2 cell monolayer adhered to the following order; neutral $\geq$ positive > negative (Pauletti et al., 1997). There is a report using a series of cyclic arginine–glycine–aspartic acid (RGD) peptides which demonstrated that peptides with a net charge of -1 to -2 exhibit optimum permeability by the paracellular route when evaluated for their transport across a Caco-2 monolayer (Rubas et al., 1994). Another study using small anionic, neutral, and cationic non-peptide markers demonstrated a higher paracellular permeability for the cationic species when evaluated for their transport across a Caco-2 cell monolayer (Adson et al., 1994). Still other research reports demonstrate no selectivity based on net charge for the flux of several model polypeptides across a Caco-2 cell monolayer (Knipp et al., 1995; Okumu et al., 1995).

Although tight junctions appear cation-selective for small molecules, the effect of overall ionic charge of a protein or polypeptide on its paracellular permeation is not known. An investigation with ficoll and ficoll sulfate, representing neutral and negatively charged spherical probes, respectively, demonstrated no significant difference between their sieving curves in an isolated glomerular basement membrane (Bolton et al., 1998). The importance of sieving curves for molecules of varying size and charge lies in their ability to indicate the mechanism by which glomerular capillary walls selectively filter plasma solutes (Bolton et al., 1998). Similar results were also observed with negatively charged dextran sulfate and neutral dextran, in which only a slight reduction in the sieving curve was detected for the negatively charged polymer (Bray and Robinson, 1984). Bolton et al. (1998) attributes this particular sieving behavior to the fact that there are not enough fixed negative charges in the glomerular basement membrane to produce appreciable charge selectivity. In other words, the "*charge density is insufficient to be functionally important*". The question, however, is whether the same conclusion may be extended to the tight junctions of the intestinal epithelium.

Our previous work with negatively charged PDGlu may serve to shed light on the effect of overall ionic charge of a polypeptide and its paracellular diffusion (Salamat-Miller et al., 2005). Assuming that each side chain of each amino acid in PDGlu contributes a charge of -1, the total charge of our model polypeptide was approximately -176 (Salamat-Miller et al., 2005). Pappenheimer and Reiss (1987) studied the paracellular permeation of negatively charged ferrocyanide ion (-4) across rat small intestine and did not observe any appreciable transport of this molecule despite its small molecular weight (212 Da). Our findings with highly negatively charged PDGlu (-176) suggest that either the charge-to-mass ratio or the density of the fixed negative charges on the intestinal epithelium may be playing a role in its overall paracellular transport. Either or both of these two factors can potentially affect the paracellular transport of a polypeptide. If the ratio of charge to mass is low or the concentration of the fixed negative charges on the intestinal membrane is not great enough to cause a significant repulsion of PDGlu, then PDGlu would be able to permeate through the negatively charged tight junctions. We suggested that the influence of the negatively charged carboxylate groups associated with the RC conformation of PDGlu exerted a negligible effect with regard to paracellular diffusion through the negatively charged tight junctions (Salamat-Miller et al., 2005). This result is similar to the conclusion advanced by Pauletti et al. (1997) with regard to the charge selectivity of tight junctions; namely, that the contribution of net charge to overall transport at the level of a hexapeptide or greater is virtually negligible.

As stated above, the effect of overall ionic charge on the paracellular transport of charged macromolecules may be related to the charge-to-mass ratio. With increasing molecular weight, the charge-to-mass ratio of a molecule decreases to such a negligible

value that additional physicochemical factors, other than charge alone, govern the diffusion process. The charge-to-mass ratio for ferrocyanide is −0.019 (=−4/212), whereas, this ratio substantially decreases to -6.6×10^{-3} (=−176/26,600) for PDGlu. Based on our findings with PDGlu, we suggested that the paracellular transport we observed across both a Caco-2 cell monolayer and rat intestinal membrane was primarily dependent on the polypeptide's capacity to undergo molecular deformation during its diffusion into and through the negatively charged tight junction (Salamat-Miller et al., 2005). That is, molecular flexibility inherent in the RC conformer of PDGlu, combined with a steep concentration gradient, appeared to 'override' any charge repulsion that might occur between the fixed negative charges of the tight junction and the rather insignificant negative value of the charge-to-mass ratio associated with PDGlu. Moreover, based on the overall dimensions of PDGlu, as reflected by a hydrodynamic radius that was much larger than the pore radius of a tight junction, we also suggested that molecular flexibility overcame the constraints of restricted diffusion as described by the Renkin molecular sieving function (Salamat-Miller et al., 2005).

6. Methods to increase paracellular drug permeation

6.1. Physicochemical modification of the permeant

This approach takes advantage of chemical and/or physical modifications of the drug molecule. The partial or total substitution of the L-isomer form of amino acids with the D-isomer form has been used to overcome enzymatic degradation by gastrointestinal luminal enzymes (Pappenheimer et al., 1994, 1997; He et al., 1996; Tamura et al., 1996, 1997). Several peptides synthesized with the D-isomer have been shown to be potent substitutes for naturally occurring peptide hormones or antibiotics of clinical interest. For instance, Dooley et al. (1994) has demonstrated the central analgesic activity of an all D-amino acid opioid peptide. Other successful examples of this approach include the D-isomers of naturally occurring antibiotics (Wade et al., 1990; Merrifield et al., 1995) and a β-endorphin analog containing only D-amino acids in its C-terminus (Blanc and Kaiser, 1984).

Effort to make peptides/polypeptides more lipophilic is another approach to manipulate the physicochemical properties of a molecule. Using this approach, the chemical properties of the peptide are intentionally altered so that the molecule can be made to undergo passive diffusion by the transcellular route (Pauletti et al., 1996b; Knipp et al., 1997; Okumu et al., 1997; Bak et al., 1999a, 1999b; Gudmundsson et al., 1999a; Wang et al., 1999). This approach typically involves the attachment of lipophilic functional groups to the molecule of interest. Since this strategy is aimed at facilitating the absorption of drug substances by the transcellular pathway, it will not be further discussed.

A more recent approach to altering the physicochemical properties of protein and polypeptide drugs is the use of delivery agents. These agents are low-molecular-weight, peptide-like compounds that either covalently or non-covalently form a complex with the protein or polypeptide drug in order to increase its oral bioavailability. This would appear to be a very promising approach for the oral delivery of protein and polypeptide drugs. Examples include the successful oral delivery of salmon calcitonin (sCT) and interferon-α in rats and primates with the aid of low-molecular-weight acylated amino acids (Leone-Bay et al., 1995). In this investigation, a positive correlation was demonstrated between log *P* of the delivery agents and the extent of oral absorption of sCT in rats. However, this relationship was less well-defined for the intestinal absorption of interferon-α. The more complex tertiary structure of interferon-α was suggested to contribute to the ambiguous relationship between log *P* of the delivery agent and the oral bioavailability of interferon-α (Leone-Bay et al., 1995). Other delivery agents, such as *N*-acylated, non-α-amino acids, have been used to improve the oral absorption of recombinant human growth hormone (rhGH) in rats and primates (Leone-Bay et al., 1996). In that study, a direct relationship was demonstrated between the lipophilicity of the agent and the fraction of rhGH absorbed following an oral dose (Leone-Bay et al., 1996).

Oral delivery of biologically active parathyroid hormone (PTH) has also been reported with the aid of delivery agents (Leone-Bay et al., 2001). In this study, a PTH/delivery agent complex was orally administered in a rat model of osteoporosis and demonstrated comparable efficacy to parenteral administration of the hormone (Leone-Bay et al., 2001). Although non-covalent

interactions between specific delivery agents and a protein/polypeptide drug clearly occur and cause conformational changes in the native structure of the protein, the nature of the conformational change(s) (folding versus unfolding versus misfolding) is not known (Leone-Bay et al., 2001). These authors demonstrated that the enhanced oral bioavailability observed with the PTH/delivery agent complex was not due to either inhibition of intestinal enzymes by the complex or damage to the membrane (Leone-Bay et al., 1995). Because the delivery agents contained in these complexes are lipophilic, it is entirely possible that the transcellular pathway was involved in the permeation process due to exposure of the protein's hydrophobic side chains (Milstein et al., 1998). However, paracellular transport of the PTH/delivery agent complex cannot be conclusively ruled out as a mechanism for the enhanced oral absorption of the complex (Sood and Panchagnula, 2001). Regardless of the nature of the conformational changes that occur in the protein by the delivery agents, the resulting complexes demonstrated an enhanced permeation across the GI tract and are clearly worthy of further investigation.

In addition to non-covalently bound protein-delivery agent complexes, a strategy based on covalent interactions has been reported to increase the oral delivery of proteins. In this approach, the structure of a protein is chemically modified by attaching amphiphilic oligomers to specific sites on the molecule (Still, 2002). These oligomers are composed of lipophilic alkyl chains and hydrophilic polyethylene units. One specific example of this type of protein modification is conjugated hexyl insulin (HIM2). In this complex, a single oligomer consisting of 7–9 units of PEG and a hexyl alkyl chain is covalently attached to lysine residue 29 on the β-chain of recombinant human insulin (Still, 2002). Using conjugated insulin, Still (2002) demonstrated greater stability against enzymatic degradation (2–10 times more stable), improved solubility (2–8 times more soluble), and a prolonged circulation half-life. Receptor binding of monoconjugated hexyl-insulin was approximately 70% of the native insulin and its glucose-lowering effect in both normal volunteers and Type-1 and Type-2 diabetic patients was significant (Still, 2002). The effect of HIM2 has now been investigated in limited clinical studies involving both Type-1 and Type-2 diabetic patients (Kipnes et al., 2003; Clement et al., 2004).

6.2. Modulating the tight junctions associated with the paracellular pathway

An alternative to physicochemical modification of the permeant is manipulation of the structure of tight junctions to expand the pore diameter and increase the amount of the candidate macromolecule that undergoes paracellular absorption. Compounds that modulate tight junctions, such as calcium chelating agents, surfactants, medium-chain fatty acids, bile salts, phosphate esters, and some cationic polymers, have been extensively described in the literature (Ward et al., 2000; Sood and Panchagnula, 2001). These compounds act through three major mechanisms to modulate the paracellular pathway; namely, their effect(s) on the (1) mucous layer, (2) membrane components, and/or (3) tight junctions (Junginger and Verhoef, 1998). It should be mentioned that many of the perturbants used to modulate (reversibly expand) the tight junctions, including EDTA and EGTA, have been suggested to function by altering protein kinase-C (PKC) signaling (Citi, 1992; Tomita et al., 1996). These studies demonstrated that removal of extracellular calcium by calcium chelators activates PKC. It is thought that PKC activation is mediated by the calcium-dependent, cell adhesion protein, cadherin. Specifically, Noach et al. (1993) suggested that depletion of extracellular calcium by various chelators caused uvomorulin (a member of the cadherin family) to be internalized. Internalization of uvomorulin triggers a cascade of cellular signaling, which disassembles all cytoskeleton/junctional components, including perijunctional actin rings, ZO-1, and, possibly desmosomes (Citi, 1992; Cereijido et al., 1993).

These exogenously added compounds are usually non-specific, toxic, and damaging to the mucosal lining of the intestinal membrane (Ward et al., 2000). However, new permeation enhancers for protein and polypeptide drugs which act through different mechanisms and without the side-effects of conventional permeation enhancers have been introduced. Among these recently introduced penetration enhancers, zonula occluden toxin (ZOT) has proven to be the most attractive alternative (Fasano and Uzzau, 1997; Fasano, 1998b; Salama et al., 2003). ZOT is neither cytotoxic nor damaging to intestinal epithelial cells ex vivo (Fasano, 1998b). It increases the paracellular permeability of a variety of compounds by reversibly expanding tight junctions. ZOT interacts with specific receptors located

primarily within the small intestine. It is believed that ZOT expands tight junctions through PKC-induced effects on the actin cytoskeleton of the cell (Fasano et al., 1995). The specific binding of ZOT to intestinal receptors also prevents colonic microflora from entering the systemic circulation (Fasano, 1998b). Although using ZOT is very attractive for preventing the possible entrance of toxins into the systemic circulation, the possibility of an immunogenic compound reaching the circulation cannot be excluded. This concern brings into question the safety of using ZOT to modulate tight junctions for the oral delivery of therapeutic proteins and polypeptides.

Other 'relatively safe' permeation enhancers, such as anionic polyacrylate derivatives and cationic chitosan derivatives, have been shown to have no acute toxicity and cell damaging effects to Caco-2 cell monolayers (Junginger and Verhoef, 1998). It is suggested that upon exposure of intestinal epithelial cells to anionic polyacrylate derivatives, a complex between endogenous calcium from the intestinal cells and poly(acrylic acid) is formed. By removing endogenous calcium from the intestinal epithelial cells, tight junctions expand. This effect has been demonstrated using Caco-2 cell monolayers (Lueßen et al., 1994). Poly(acrylic acid) can also absorb water from the cells. Water absorption by a dry and swellable polymer results in cell dehydration and subsequent cell shrinking, which ultimately results in expansion of the spaces between the cells (Haas and Lehr, 2002).

Chitosan derivatives, such as *N*-trimethyl chitosan chloride, increase the paracellular transport of drug compounds by a different mechanism. The interaction between the positive charges of cationic chitosan and anionic glycoproteins on the surface of the epithelial cells can displace specific cations necessary for the coordinated closing of tight junctions (Kotze et al., 1999). It has also been shown that chitosan derivatives can redistribute actin filaments and reorganize the protein structure of tight junctions, thus enhancing the paracellular transport of various paracellular marker molecules (Dodane et al., 1999).

Thiolated polymers, or thiomers, are another recently introduced category of permeation enhancers that may potentially increase the paracellular transport of a variety of drug compounds. Thiomers have proven effective in the transmucosal delivery of protein and polypeptides; specifically, across the buccal epithelium (Kast and Bernkop-Schnürch, 2001; Marschutz and Bernkop-Schnürch, 2002). Recently, a new enhancer consisting of a thiolated polymer [poly(acrylic acid)-cysteine, chitosan-4-thio-butylamidine] and reduced glutathione (GSH) has been shown to increase the paracellular transport of sCT, insulin, and heparin across rat intestinal epithelium in vivo and freshly excised guinea pig intestinal mucosa in vitro (Bernkop-Schnürch et al., 2003). The permeation-enhancing effect of this system has been attributed to inhibition of protein tyrosine phosphatase (PTP). This enzyme dephosphorylates the tyrosine residues on occludins located in the tight junctions and serves to close the junctions. Inhibition of PTP by the thiomer/GSH system causes the tight junctions to expand, which ultimately leads to enhanced paracellular transport of hydrophilic compounds through the tight junctions (Bernkop-Schnürch et al., 2003).

A proline-containing, hydrophilic, and collagenase-labile pentapeptide, known as Pz-peptide (4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L - Pro-D-Arg), has demonstrated transient and reversible opening of tight junctions in Caco-2 cell monolayers, as well as rabbit intestinal membranes (Yen and Lee, 1995a). In a concentration-dependent manner, Pz-peptide demonstrated an ability to not only increase its own paracellular transport, but also increased the transport of several paracellular markers (Yen and Lee, 1994). When Pz-peptide was compared with more common penetration enhancers, such as EDTA and cytochalasin B, there was a 300-fold reduction in the expansion of tight junctions (Yen and Lee, 1995a). The authors interpreted this finding as meaning that Pz-peptide was less perturbing to the tight junctions compared to more conventional permeation enhancers (Yen and Lee, 1995a). Additionally, this peptide exhibited a site-specific permeation pattern, with similar paracellular transport properties in the duodenum and jejunum and higher permeation in the colon and rectum (Yen and Lee, 1994). The pentapeptide studied by Yen and Lee (1995b) has been found to stimulate transepithelial Na^+ flux across colonic segments at amiloride-sensitive Na^+ channels and triggers tight junction opening through unknown cellular mechanisms. The particular chemical structure and the unusual mechanism of action of Pz-peptide could possibly be utilized in designing therapeutic peptides that would exhibit both enhanced paracellular

transport and reduced susceptibility to enzymatic inactivation.

Glucose solutions have also been shown to expand tight junctions. The effect of glucose on the transmucosal absorption of both fluid and cationic drugs was reported by Oschenfahrt and Winne (1973) and Kitazawa et al. (1975). Kitazawa et al. (1975, 1978) found that luminal concentrations of D-glucose (between 150 and 300 mM) perfused at 5.0 ml/min through the small intestine of rats enhanced the transmucosal absorption of various model drugs. In 1987, Pappenheimer, Madara, and Reiss proposed a mechanism for the Na^+-cotransported-nutrient effect that caused the expansion of tight junctions (Madara and Pappenheimer, 1987; Pappenheimer, 1987; Pappenheimer and Reiss, 1987). This theory suggested that the active transcellular transport of nutrients after a meal initiates the opening of tight junctions via osmotic force, which stimulates the flow of water through the paracellular pathway. The significance of bulk water movement lies in its capacity to carry dissolved solutes in the convective stream. An increase in bulk water flow and the paracellular transport of markers across tight junctions is known as 'solvent drag', in which the absorption of water across the intestinal epithelium is increased by either hypotonicity or Na^+-glucose cotransport of nutrients.

The concept of 'solvent drag' should be recognized as a specific driving force for the paracellular transport of hydrophilic polypeptides and macromolecules. A high nutrient content in the gut lumen activates the Na^+-glucose cotransporter, which is followed by cell swelling and an intercellular phosphorylation cascade. This, in turn, results in perijunctional cytoskeleton condensation and a subsequent expansion of tight junctions (Karlsson et al., 1999; Turner and Madara, 2001). Although the theory of tight junction regulation mediated by Na^+-glucose cotransport has been demonstrated to be valid for increasing the paracellular transport of hydrophilic markers in humans (Turner et al., 2000), Caco-2 cell monolayers (Karlsson et al., 1999), and rats (Zhang and Castro, 1992), several other studies in humans (Fagerholm et al., 1999), and dogs (Lane et al., 1999) have not demonstrated this effect. It would appear that the experimental method, the infusion rate, the length and the corresponding absorptive surface area of the isolated intestinal segment, the presence of sufficient water to produce convective bulk flow, the size of the permeant(s), the differences between species, and, as yet, undefined forces and resistances related to convective water flow through the paracellular pathway, have all contributed to different experimental results from various laboratories.

Another recent approach which has been used to modulate tight junctions (i.e. expand the aqueous-filled pores of the paracellular pathway) is the use of synthetic peptides derived from either the sequence of contact (bulge and groove) regions in the extracellular domain of E-cadherin or the second putative extracellular domain of occludin (Wong and Gumbiner, 1997; Makagiansar et al., 2001; Sinaga et al., 2002). In these experiments, a conserved sequence of amino acids in the extracellular domain of either E-cadherin or occludin was selected and peptides with similar amino acid sequences were synthesized. The objective in these studies was to either inhibit the interaction between E-cadherin proteins (Makagiansar et al., 2001; Sinaga et al., 2002) or prevent polymerization of occludin contained in the transmembrane fibrils (Wong and Gumbiner, 1997), respectively, so as to impede cell-to-cell adhesion and increase the paracellular porosity of the tight junction. Several of the synthetic peptides were able to expand the tight junctions, which was subsequently confirmed by decreased TEER values and increased permeation of a paracellular marker across a monolayer of either MDCK cells (Makagiansar et al., 2001; Sinaga et al., 2002) or *Xenopus* kidney epithelial A6 cells (Wong and Gumbiner, 1997). Therefore, targeting a specific synthetic peptide to a complementary amino acid sequence contained in the extracellular domain of either E-cadherin or occludin represents an innovative approach to increasing drug absorption by reversible expansion of tight junctions.

Intestinal tight junctions may also be modulated without the influence of exogenous compounds, but instead, by innate cell signaling events. Physiological factors have been shown to regulate signaling events in the cell by affecting two major pathways. These events affect either the cytoskeleton-mediated regulation of tight junctions or tight junction proteins or both (Hecht, 2001). Additional evidence has demonstrated 'cross-talk' between these two regulatory pathways, where specific tight junction proteins interact with various microfilaments within the perijunctional actomyosin (Madara, 1987). Concentration of cyclic AMP (Duffey et al., 1981), intercellular calcium con-

centrations (Guo et al., 2003), small GTPases such as *Rho* and *Rac* (Ridley and Hall, 1992; Ridley et al., 1992; Jou et al., 1998), and phosphorylation of myosin's light-chain (Turner et al., 1997) are some of the physiological factors that exert a regulatory effect on either tight junction proteins or actin.

The strategy of reversibly expanding tight junctions to increase the paracellular transport of drug molecules is not without safety concerns. Because the expansion of tight junctions introduces the potential for absorption of unwanted toxins/bacteria/immunogens into the systemic circulation, the application and safety of all the approaches described above is still questionable. Thus, the importance of modifying the physicochemical properties of the permeant may represent a more practical and safer strategy for increasing the paracellular absorption of therapeutic proteins and polypeptides.

7. Concluding remarks

The influence of overall geometry of a therapeutic protein or polypeptide on its passive, hindered (restricted) paracellular diffusion is very complex. The importance of a molecule's overall shape or geometry on its diffusion through a porous barrier has long been recognized in the chemical engineering literature. However, in the pharmaceutical sciences, the importance of molecular geometry on hindered and non-hindered aqueous diffusion has not been emphasized, particularly for macromolecules such as proteins and extended polypeptides. This may result from the fact that most traditional/conventional drug substances fall within a molecular weight range of approximately 100–750 g/mol. Considering the fact that protein drugs represented about one-third of all FDA-approved drugs in 2002 (Szymkowski, 2004), much more research is required to completely elucidate the affect that a polypeptide's shape or molecular dimensions has on its paracellular absorption from the intestine. It is now becoming increasingly important to recognize how a therapeutic polypeptide's structure, including its three-dimensional spatial orientation conferred from its secondary structure, relates to its biological activity. For example, aggregation of the polypeptide associated with Alzheimer's disease, β-amyloid peptide (Aβ), results from the transition of a random coil structure in the native state to a β-sheet conformation. The β-sheet secondary structure of Aβ precipitates and forms the neuritic plaques observed in the brain of these patients (Otvos et al., 1993; El-Agnaf et al., 1998). Depending on the solvent, Aβ can change from either an unordered or α-helix structure to a parallel or antiparallel β-sheet structure in fibrils (Tjernberg et al., 2002). Another example of a protein conformational disease is the formation of cataracts (Crabbe, 1998).

Currently, the rational design of protein drugs utilizes specific structure modifications in order to optimize pharmacokinetic and physicochemical properties. Extensive research conducted by Leone-Bay et al., (1995, 1996, 2001) has demonstrated the importance of the overall geometry of a therapeutic protein on its oral absorption. By inducing conformational changes, this research group has demonstrated enhanced oral absorption for various protein and polypeptide drugs, although the mechanism(s) responsible for improved absorption are still unknown. It may be that, upon binding to specific delivery agents, the protein drug undergoes a conformational change to a molten globule state with increased flexibility, which, in turn, increases both the rate and extent of paracellular transport across the intestinal epithelium. However, it should be emphasized that this proposed mechanism is only a suggestion by the present authors.

Investigation of the underlying mechanism(s) responsible for enhanced paracellular transport that may be achieved by intentionally inducing a change in the conformation of a polypeptide drug will require much additional research by scientists in a variety of disciplines. To meet this goal, we propose that research efforts be directed toward investigating the effect of such factors as the overall geometry and spatial orientation of a therapeutic polypeptide (conferred from its secondary structure) as it approaches the entrance to the tight junction, the potential for deformation while undergoing paracellular transport, and even the possibility of exploiting membrane transporters to recognize and selectively bind polypeptides possessing a specific three-dimensional shape/geometry so as to increase their intestinal absorption. Work in our laboratory has conclusively shown that the secondary structure associated with a model polypeptide did indeed influenced both the rate and extent of its paracellular transport through tight junctions (Salamat-Miller et al., 2005)

and opens up the exciting field of secondary structure-dependent paracellular transport of polypeptide drugs.

References

Adson, A., Raub, T.J., Burton, P.S., Barsuhn, C.L., Hilgers, A.R., Audus, K.L., Ho, N.F., 1994. Quantitative approaches to delineate paracellular diffusion in cultured epithelial cell monolayers. J. Pharm. Sci. 83, 1529–1536.

Anderson, J.M., Cereijido, M., 2001. Introduction: evolution of ideas on the tight junction. In: Cereijido, M., Anderson, J. (Eds.), Tight Junctions, second ed. CRC Press, Boca Raton, pp. 1–18.

Artursson, P., Ungell, A.L., Löfroth, J.E., 1993. Selective paracellular permeability in two models of intestinal absorption: cultured monolayers of human intestinal epithelial cells and rat intestinal segments. Pharm. Res. 10, 1123–1129.

Bak, A., Gudmundsson, O.S., Friis, G.J., Siahaan, T.J., Borchardt, R.T., 1999a. Acyloxyalkoxy-based cyclic prodrugs of opioid peptides: evaluation of the chemical and enzymatic stability as well as their transport properties across Caco-2 cell monolayers. Pharm. Res. 16, 24–29.

Bak, A., Siahaan, T.J., Gudmundsson, O.S., Gangwar, S., Friis, G.J., Borchardt, R.T., 1999b. Synthesis and evaluation of the physicochemical properties of esterase-sensitive cyclic prodrugs of opioid peptides using an (acyloxy)alkoxy linker. J. Pept. Res. 53, 393–402.

Bernkop-Schnürch, A., Kast, C.E., Guggi, D., 2003. Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems. J. Control. Release 93, 95–103.

Blanc, J.P., Kaiser, E.T., 1984. Biological and physical properties of a beta-endorphin analog containing only D-amino acids in the amphiphilic helical segment 13-31. J. Biol. Chem. 259, 9549–9556.

Bock, U., Kolac, C., Borchard, G., Koch, K., Fuchs, R., Streichhan, P., Lehr, C., 1998. Transport of proteolytic enzymes across Caco-2 cell monolayers. Pharm. Res. 15, 1393–1400.

Bohrer, M.P., Deen, W.M., Robertson, C.R., Troy, J.L., Brenner, B.M., 1979. Influence of molecular configuration on the passage of macromolecules across the glomerular capillary wall. J. Gen. Physiol. 74, 583–593.

Bohrer, M.P., Patterson, G.D., Carroll, P.J., 1984. Hindered diffusion of dextran and ficoll in microporous membranes. Macromolecules 17, 1170–1173.

Bolton, G.R., Deen, W.M., Daniels, B.S., 1998. Assessment of the charge selectivity of glomerular basement membrane using ficoll sulfate. Am. J. Physiol. 274, F889–F896.

Bray, J., Robinson, G.B., 1984. Influence of charge on filtration across renal basement membrane films in vitro. Kidney Int. 25, 527–533.

Burns, A.R., Walker, D.C., Smith, C.W., 2001. Relationship between tight junctions and leukocyte transmigration. In: Cereijido, M., Anderson, J. (Eds.), Tight Junctions, second ed. CRC Press, Boca Raton, pp. 629–652.

Cereijido, M., Gonzalez-Mariscal, R.G., Contreras, R.G., Garcia-Villegas, R., Valdes, J., 1993. The making of a tight junction. J. Cell Sci. 17, 127–132.

Citi, S., 1992. Protein kinase inhibitors prevent junction dissociation induced by low extracellular calcium in MDCK epithelial cells. J. Cell Biol. 117, 169–178.

Claude, P., 1978. Morphological factors influencing transepithelial permeability: a model for the resistance of the *zonula occludens*. J. Membr. Biol. 39, 219–232.

Claude, P., Goodenough, D.A., 1973. Fracture faces of zonulae occludentes from "tight" and "leaky" epithelia. J. Cell Biol. 58, 390–400.

Clement, S., Dandona, P., Still, J.G., Kosutic, G., 2004. Oral modified insulin (HIM2) in patients with type 1 diabetes mellitus: results from a phase I/II clinical trial. Metabolism 53, 54–58.

Collett, A., Sims, E., Walker, D., He, Y.L., Ayrton, J., Rowland, M., Warhurst, G., 1996. Comparison of HT29-18-C1 and Caco-2 cell lines as models for studying intestinal paracellular drug absorption. Pharm. Res. 13, 216–221.

Crabbe, M.J., 1998. Cataract as a conformational disease—the Maillard reaction (α-crystallin and chemotherapy. Cell Mol. Biol. (Noisy-le-grand) 44, 1047–1050.

Dodane, V., Amin Khan, M., Merwin, J.R., 1999. Effect of chitosan on epithelial permeability and structure. Int. J. Pharm. 182, 21–32.

Dodoo, A.N., Bansal, S., Barlow, D.J., Bennet, F.C., Hider, R.C., Lansley, A.B., Lawrence, M.J., Marriott, C., 2000. Systematic investigations of the influence of molecular structure on the transport of peptides across cultured alveolar cell monolayers. Pharm. Res. 17, 7–14.

Dooley, C.T., Chung, N.N., Wilkes, B.C., Schiller, P.W., Bidlack, J.M., Pasternak, G.W., Houghten, R.A., 1994. An all D-amino acid opioid peptide with central analgesic activity from a combinatorial library. Science 266, 2019–2022.

Drewe, J., Fricker, G., Vonderscher, J., Beglinger, C., 1993. Enteral absorption of octreotide: absorption enhancement by polyoxyethylene-24-cholesterol ether. Br. J. Pharmacol. 108, 298–303.

Duffey, M.E., Hainau, B., Ho, S., Bentzel, C.J., 1981. Regulation of epithelial tight junction permeability by cyclic AMP. Nature 294, 451–453.

El-Agnaf, O.M., Guthrie, D.J., Walsh, D.M., Irvine, G.B., 1998. The influence of the central region containing residues 19-25 on the aggregation properties and secondary structure of Alzheimer's beta-amyloid peptide. Eur. J. Biochem. 256, 560–569.

El-Sayed, M., Kiani, M.F., Naimark, M.D., Hikal, A.H., Ghandehari, H., 2001. Extravasation of poly(amidoamine) (PAMAM) dendrimers across microvascular network endothelium. Pharm. Res. 18, 23–28.

Fagerholm, U., Nilsson, D., Knutson, L., Lennernäs, H., 1999. Jejunal permeability in humans in vivo and rats in situ: investigation of molecular size selectivity and solvent drag. Acta Physiol. Scand. 165, 315–324.

Fasano, A., 1998a. Modulation of intestinal permeability: an innovative method of oral drug delivery for the treatment of inherited and acquired human diseases. Mol. Genet. Metab. 64, 12–18.

Fasano, A., 1998b. Novel approaches for oral delivery of macromolecules. J. Pharm. Sci. 87, 1351–1356.

Fasano, A., Fiorentini, C., Donelli, G., Uzzau, S., Kaper, J.B., Margaretten, K., Ding, X., Guandalini, S., Comstock, L., Goldblum, S.E., 1995. *Zonula occludens* toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. J. Clin. Invest. 96, 710–720.

Fasano, A., Uzzau, S., 1997. Modulation of intestinal tight junctions by *zonula occludens* toxin permits enteral administration of insulin and other macromolecules in an animal model. J. Clin. Invest. 99, 1158–1164.

Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K., Tsukita, S., 1998. Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. J. Cell Biol. 141, 1539–1550.

Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S., 1993. Occludin: a novel integral membrane protein localizing at tight junctions. J. Cell Biol. 123, 1777–1788.

Gardner, M.L.G., 1984. Intestinal assimilation of intact peptides and proteins from the diet-A neglected field? Biol. Rev. 59, 289–331.

González-Mariscal, L., Avila, A., Betanzos, A., 2001. The relationship between structure and function of tight junctions. In: Cereijido, M., Anderson, J. (Eds.), Tight Junctions, second ed. CRC Press, Boca Raton, pp. 89–119.

Gudmundsson, O.S., Jois, S.D., Vander Velde, D.G., Siahaan, T.J., Wang, B., Borchardt, R.T., 1999a. The effect of conformation on the membrane permeation of coumarinic acid- and phenylpropionic acid-based cyclic prodrugs of opioid peptides. J. Pept. Res. 53, 383–392.

Guo, X., Rao, J.N., Liu, L., Zou, T.T., Turner, D.J., Bass, B.L., Wang, J.Y., 2003. Regulation of adherens junctions and epithelial paracellular permeability: a novel function for polyamines. Am. J. Physiol. Cell Physiol. 285, 1174–1187.

Haas, J., Lehr, C.M., 2002. Developments in the area of bioadhesive drug delivery systems. Expert Opin. Biol. Ther. 2, 287–298.

Hamilton, I., Cobden, I., Axon, A.T., 1984. In vitro determination of small intestinal permeability. Gut 25, 909–910.

He, Y.L., Murby, S., Gifford, L., Collett, A., Warhurst, G., Douglas, K.T., Rowland, M., Ayrton, J., 1996. Oral absorption of D-oligopeptides in rats via the paracellular route. Pharm. Res. 13, 1673–1678.

Hecht, G., 2001. Microbial pathogens that affect tight junctions. In: Cereijido, M., Anderson, J. (Eds.), Tight Junctions, second ed. CRC Press, Boca Raton, pp. 493–515.

Hollander, D., Ricketts, D., Boyd, C.A.R., 1988. Importance of "probe" molecular geometry in determining intestinal permeability. Can. J. Gastroentrol. 2, 35A–38A.

Horibe, Y., Hosoya, K., Kim, K.J., Ogiso, T., Lee, V.H., 1997. Polar solute transport across the pigmented rabbit conjunctiva: size dependence and the influence of 8-bromo cyclic adenosine monophosphate. Pharm. Res. 14, 1246–1251.

Jaehde, U., Masereeuw, R., De Boer, A.G., Fricker, G., Nagelkerke, J.F., Vonderscher, J., Breimer, D.D., 1994. Quantification and visualization of the transport of octreotide, a somatostatin analogue, across monolayers of cerebrovascular endothelial cells. Pharm. Res. 11, 442–448.

Johnson Jr., W.C., Tinoco Jr., I., 1972. Circular dichroism of polypeptide solutions in the vacuum ultraviolet. J. Am. Chem. Soc. 94, 4389–4392.

Jou, T.S., Schneeberger, E.E., Nelson, W.J., 1998. Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. J. Cell Biol. 142, 101–115.

Junginger, H.E., Verhoef, J.C., 1998. Macromolecules as safe penetration enhancers for hydrophilic drugs—A fiction? Pharm. Sci. Technol. Today 1, 370–376.

Karlsson, J., Ungell, A., Gråsjö, J., Artursson, P., 1999. Paracellular drug transport across intestinal epithelia: influence of charge and induced water flux. Eur. J. Pharm. Sci. 9, 47–56.

Kast, C.E., Bernkop-Schnürch, A., 2001. Thiolated polymers—thiomers: development and in vitro evaluation of chitosan-thioglycolic acid conjugates. Biomaterials 22, 2345–2352.

Kipnes, M., Dandona, P., Tripathy, D., Still, J.G., Kosutic, G., 2003. Control of postprandial plasma glucose by an oral insulin product (HIM2) in patients with type 2 diabetes. Diabetes Care 26, 421–426.

Kitazawa, S., Ito, H., Johno, I., Takahashi, T., Takenaka, H., 1978. Generality in effects of transmucosal fluid movement and glucose on drug absorption from the rat small intestine. Chem. Pharm. Bull. (Tokyo) 26, 915–924.

Kitazawa, S., Ito, H., Sezaki, H., 1975. Transmucosal fluid movement and its effect on drug absorption. Chem. Pharm. Bull. (Tokyo) 23, 1856–1865.

Knipp, G.T., Vander Velde, D., Siahaan, T.J., Borchardt, R.T., 1995. The effect of solution conformation and charge on the paracellular permeability of model pentapeptides across Caco-2 cell monolayer. Pharm. Res. 12, S-303.

Knipp, G.T., Vander Velde, D.G., Siahaan, T.J., Borchardt, R.T., 1997. The effect of β-turn structure on the passive diffusion of peptides across Caco-2 cell monolayers. Pharm. Res. 14, 1332–1340.

Kobayashi, S., Kondo, S., Juni, K., 1995. Permeability of peptides and proteins in human cultured alveolar A549 cell monolayer. Pharm. Res. 12, 1115–1119.

Kotze, A.F., Thanou, M.M., Luebetaen, H.L., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1999. Enhancement of paracellular drug transport with highly quaternized *N*-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2). J. Pharm. Sci. 88, 253–257.

Lane, J.S., Whang, E.E., Rigberg, D.A., Hines, O.J., Kwan, D., Zinner, M.J., McFadden, D.W., Diamond, J., Ashley, S.W., 1999. Paracellular glucose transport plays a minor role in the unanesthetized dog. Am. J. Physiol. 276, 789–794.

Lane, M.E., O'Driscoll, C.M., Corrigan, O.I., 1996. The relationship between rat intestinal permeability and hydrophilic probe size. Pharm. Res. 13, 1554–1558.

Lang, V.B., Langguth, P., Ottiger, C., Wunderli-Allenspach, H., Rognan, D., Rothen-Rutishauser, B., Perriard, J.C., Lang, S., Biber, J., Merkle, H.P., 1997. Structure-permeation relations of metenkephalin peptide analogues on absorption and secretion mechanisms in Caco-2 monolayers. J. Pharm. Sci. 86, 846–853.

Lee, Y.H., Sinko, P.J., 2000. Oral delivery of salmon calcitonin. Adv. Drug Deliv. Rev. 42, 225–238.

Lennernäs, H., Nylander, S., Ungell, A.L., 1997. Jejunal permeability: A comparison between the using chamber technique and the single-pass perfusion in humans. Pharm. Res. 14, 667–671.

Leone-Bay, A., Ho, K.K., Agarwal, R., Baughman, R.A., Chaudhary, K., DeMorin, F., Genoble, L., McInnes, C., Lercara, C., Milstein, S., O'Toole, D., Sarubbi, D., Variano, B., Paton, D.R., 1996. 4-[4-[(2-Hydroxybenzoyl)amino]phenyl] butyric acid as a novel oral delivery agent for recombinant human growth hormone. J. Med. Chem. 39, 2571–2578.

Leone-Bay, A., Santiago, N., Achan, D., Chaudhary, K., DeMorin, F., Falzarano, L., Haas, S., Kalbag, S., Kaplan, D., Leipold, H., et al., 1995. *N*-acylated α-amino acids as novel oral delivery agents for proteins. J. Med. Chem. 38, 4263–4269.

Leone-Bay, A., Sato, M., Paton, D., Hunt, A.H., Sarubbi, D., Carozza, M., Chou, J., McDonough, J., Baughman, R.A., 2001. Oral delivery of biologically active parathyroid hormone. Pharm. Res. 18, 964–970.

Lueßen, H.L., Lehr, C.M., Rentel, C.O., Noach, A.B.J., de Boer, A.G., Verhoef, J.C., Junginger, H.E., 1994. Bioadhesive polymers for the peroral delivery of peptide drugs. J. Control. Release 29, 329–338.

Lundin, S., Artursson, P., 1990. Absorption of a vasopressin analogue, 1-deamino-8-D-arginine-vasopressin (dDAVP), in human intestinal epithelial cell line, Caco-2. Int. J. Pharm. 64, 181–186.

Madara, J.L., Barenberg, D., Carlson, S., 1986. Effects of cytochalasin D on occluding junctions of intestinal absorptive cells: further evidence that the cytoskeleton may influence paracellular permeability and junctional charge selectivity. J. Cell Biol. 102, 2125–2136.

Madara, J.L., 1987. Intestinal absorptive cell tight junctions are linked to cytoskeleton. Am. J. Physiol. 253, C171–C175.

Madara, J.L., Pappenheimer, J.R., 1987. Structural basis for physiological regulation of paracellular pathways in intestinal epithelia. J. Membr. Biol. 100, 149–164.

Makagiansar, I.T., Avery, M., Hu, Y., Audus, K.L., Siahaan, T.J., 2001. Improving the selectivity of HAV-peptides in modulating E-cadherin-E-cadherin interactions in the intercellular junction of MDCK cell monolayers. Pharm. Res. 18, 446–453.

Marschutz, M.K., Bernkop-Schnürch, A., 2002. Thiolated polymers: self-crosslinking properties of thiolated 450 kDa poly(acrylic acid) and their influence on mucoadhesion. Eur. J. Pharm. Sci. 15, 387–394.

Matsukawa, Y., Lee, V.H., Crandall, E.D., Kim, K.J., 1997. Size-dependent dextran transport across rat alveolar epithelial cell monolayers. J. Pharm. Sci. 86, 305–309.

Merrifield, R.B., Juvvadi, P., Andreu, D., Ubach, J., Boman, A., Boman, H.G., 1995. Retro and retroenantio analogs of cecropin-melittin hybrids. Proc. Natl. Acad. Sci. U.S.A. 92, 3449–3453.

Milstein, S.J., Leipold, H., Sarubbi, D., Leone-Bay, A., Mlynek, G.M., Robinson, J.R., Kasimova, M., Freire, E., 1998. Partially unfolded proteins efficiently penetrate cell membranes—implications for oral drug delivery. J. Control. Release 53, 259–267.

Nellans, H.N., 1991. Mechanism of peptide and protein absorption, paracellular intestinal transport: modification of absorption. Adv. Drug Deliv. Rev. 7, 339–364.

Noach, A.B.J., Kurosaki, Y., Blom-Roosemalen, M.C.M., de Boer, A.G., Breimer, D.D., 1993. Cell polarity dependent effect of chelation on the paracellular permeability of Caco-2 cell monolayer. Int. J. Pharm. 90, 229–235.

Okumu, F.W., Pauletti, G.M., Vander Velde, D.G., Siahaan, T.J., Borchardt, R.T., 1995. The effect of charge and conformation on the permeability of a hexapeptide across monolayers of a cultured human intestinal epithelial cell (Caco-2 cells). Pharm. Res. 12, S-302.

Okumu, F.W., Pauletti, G.M., Vander Velde, D.G., Siahaan, T.J., Borchardt, R.T., 1997. Effect of restricted conformational flexibility on the permeation of model hexapeptides across Caco-2 cell monolayers. Pharm. Res. 14, 169–175.

Oschenfahrt, H., Winne, D., 1973. The contribution of solvent drag to the intestinal absorption of tritiated water and urea from the jejunum of the rat, *N*-Schmied. Arch. Pharmacol. 279, 133–152.

Otvos Jr., L., Szendrei, G.I., Lee, V.M., Mantsch, H.H., 1993. Human and rodent Alzheimer β-amyloid peptides acquire distinct conformations in membrane-mimicking solvents. Eur. J. Biochem. 211, 249–257.

Pappenheimer, J.R., 1987. Physiological regulation of transepithelial impedance in the intestinal mucosa of rats and hamsters. J. Membr. Biol. 100, 137–148.

Pappenheimer, J.R., Dahl, C.E., Karnovsky, M.L., Maggio, J.E., 1994. Intestinal absorption and excretion of octapeptides composed of D-amino acids. Proc. Natl. Acad. Sci. U.S.A. 91, 1942–1945.

Pappenheimer, J.R., Karnovsky, M.L., Maggio, J.E., 1997. Absorption and excretion of undegradable peptides: role of lipid solubility and net charge. J. Pharmacol. Exp. Ther. 280, 292–300.

Pappenheimer, J.R., Reiss, K.Z., 1987. Contribution of solvent drag through intercellular junctions to absorption of nutrients by the small intestine in the rat. J. Membr. Biol. 100, 123–136.

Pauletti, G.M., Gangwar, S., Knipp, G.T., Nerurkar, M.M., Okumu, F.W., Tamura, K., Siahaan, T.J., Borchardt, R.T., 1996a. Structural requirements for intestinal absorption of peptide drugs. J. Control. Release 41, 3–17.

Pauletti, G.M., Gangwar, S., Okumu, F.W., Siahaan, T.J., Stella, V.J., Borchardt, R.T., 1996b. Esterase-sensitive cyclic prodrugs of peptides: evaluation of an acyloxyalkoxy promoiety in a model hexapeptide. Pharm. Res. 13, 1615–1623.

Pauletti, G.M., Okumu, F.W., Borchardt, R.T., 1997. Effect of size and charge on the passive diffusion of peptides across Caco-2 cell monolayers via the paracellular pathway. Pharm. Res. 14, 164–168.

Reuss, L., 2001. Tight junction permeability to ions and water. In: Cereijido, M., Anderson, J. (Eds.), Tight Junctions, second ed. CRC Press, Boca Raton, pp. 61–88.

Ridley, A.J., Hall, A., 1992. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70, 389–399.

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., Hall, A., 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70, 401–410.

Rubas, W., Jezyk, N., Grass, G.M., 1993. Comparison of the permeability characteristics of a human colonic epithelial (Caco-2)

cell line to colon of rabbit, monkey, and dog intestine and human drug absorption. Pharm. Res. 10, 113–118.

Rubas, W., Cromwell, M., Gadek, T., Narindray, D., Mrsny, R., 1994. Structural elements which govern the resistance of intestinal tissues to compound transport. Mater. Res. Soc. Symp. Proc. 331, 179–185.

Salama, N.N., Fasano, A., Lu, R., Eddington, N.D., 2003. Effect of the biologically active fragment of *zonula occludens* toxin, delta G, on the intestinal paracellular transport and oral absorption of mannitol. Int. J. Pharm. 251, 113–121.

Salamat-Miller, N.T., Chittchang, M., Mitra, A.K., Johnston, T.P., 2005. A randomly-coiled, high-molecular-weight polypeptide exhibits increased paracellular diffusion in vitro and in situ relative to the highly-ordered α-helix conformer. Pharm. Res. 22, 245–254.

Shah, R.B., Ahsan, F., Khan, M.A., 2002. Oral delivery of proteins: progress and prognostication. Crit. Rev. Ther. Drug Carrier Syst. 19, 135–169.

Sinaga, E., Jois, S.D., Avery, M., Makagiansar, I.T., Tambunan, U.S., Audus, K.L., Siahaan, T.J., 2002. Increasing paracellular porosity by E-cadherin peptides: discovery of bulge and groove regions in the EC1-domain of E-cadherin. Pharm. Res. 19, 1170–1179.

Sood, A., Panchagnula, R., 2001. Peroral route: an opportunity for protein and peptide drug delivery. Chem. Rev. 101, 3275–3303.

Stevenson, B.R., Anderson, J.M., Goodenough, D.A., Mooseker, M.S., 1988. Tight junction structure and ZO-1 content are identical in two strains of Madin–Darby canine kidney cells which differ in transepithelial resistance. J. Cell Biol. 107, 2401–2408.

Stewart, B.H., Chan, O.H., Lu, R.H., Reyner, E.L., Schmid, H.L., Hamilton, H.W., Steinbaugh, B.A., Taylor, M.D., 1995. Comparison of intestinal permeabilities determined in multiple in vitro and in situ models: relationship to absorption in humans. Pharm. Res. 12, 693–699.

Still, J.G., 2002. Development of oral insulin: progress and current status. Diabetes Metab. Res. Rev. 18, S29–S37.

Szymkowski, D.E., 2004. Rational optimization of proteins as drugs: a new era of 'medicinal biology'. Drug Discov. Today 9, 381–383.

Tamura, K., Agrios, K.A., Vander Velde, D., Aube, J., Borchardt, R.T., 1997. Effect of stereochemistry on the transport of Aca-linked β-turn peptidomimetics across a human intestinal cell line. Bioorg. Med. Chem. 5, 1859–1866.

Tamura, K., Bhatnagar, P.K., Takata, J.S., Lee, C.P., Smith, P.L., Borchardt, R.T., 1996. Metabolism, uptake, and transepithelial transport of the diastereomers of Val-Val in the human intestinal cell line, Caco-2. Pharm. Res. 13, 1213–1218.

Tanaka, Y., Taki, Y., Sakane, T., Nadai, T., Sezaki, H., Yamashita, S., 1995. Characterization of drug transport through tight-junctional pathway in Caco-2 monolayer: comparison with isolated rat jejunum and colon. Pharm. Res. 12, 523–528.

Thwaites, D.T., Hirst, B.H., Simmons, N.L., 1993. Passive transepithelial absorption of thyrotropin-releasing hormone (TRH) via a paracellular route in cultured intestinal and renal epithelial cell lines. Pharm. Res. 10, 674–681.

Tjernberg, L.O., Tjernberg, A., Bark, N., Shi, Y., Ruzsicska, B.P., Bu, Z., Thyberg, J., Callaway, D.J., 2002. Assembling amyloid fibrils from designed structures containing a significant amyloid β-peptide fragment. Biochem. J. 366, 343–351.

Tomita, M., Hayashi, M., Awazu, S., 1996. Absorption-enhancing mechanism of EDTA, caprate, and decanoylcarnitine in Caco-2 cells. J. Pharm. Sci. 85, 608–611.

Turner, J.R., 2000. Show me the pathway! Regulation of paracellular permeability by Na^+-glucose cotransport. Adv. Drug Deliv. Rev. 41, 265–281.

Turner, J.R., Cohen, D.E., Mrsny, R.J., Madara, J.L., 2000. Noninvasive in vivo analysis of human small intestinal paracellular absorption: regulation by Na^+-glucose cotransport. Dig. Dis. Sci. 45, 2122–2126.

Turner, J.R., Madara, J.L., 2001. Physiological regulation of tight junction permeability by Na^+-nutrient cotransport. In: Cereijido, M., Anderson, J. (Eds.), Tight Junctions, second ed. CRC Press, Boca Raton, pp. 333–347.

Turner, J.R., Rill, B.K., Carlson, S.L., Carnes, D., Kerner, R., Mrsny, R.J., Madara, J.L., 1997. Physiological regulation of epithelial tight junctions is associated with myosin light-chain phosphorylation. Am. J. Physiol. 273, 1378–1385.

Wade, D., Boman, A., Wahlin, B., Drain, C.M., Andreu, D., Boman, H.G., Merrifield, R.B., 1990. All D-amino acid-containing channel-forming antibiotic peptides. Proc. Natl. Acad. Sci. U.S.A. 87, 4761–4765.

Walter, E., Kissel, T., Raddatz, P., 1995. Transport of peptidomimetic renin inhibitors across monolayers of a human intestinal cell line (Caco-2): evidence for self-enhancement of paracellular transport route. Pharm. Res. 12, 1801–1805.

Walter, E., Janich, S., Roessler, B.J., Hilfinger, J.M., Amidon, G.L., 1996. HT29-MTX/Caco-2 cocultures as an in vitro model for the intestinal epithelium: in vitro-in vivo correlation with permeability data from rats and humans. J. Pharm. Sci. 85, 1070–1076.

Wang, B., Nimkar, K., Wang, W., Zhang, H., Shan, D., Gudmundsson, O., Gangwar, S., Siahaan, T., Borchardt, R.T., 1999. Synthesis and evaluation of the physicochemical properties of esterase-sensitive cyclic prodrugs of opioid peptides using coumarinic acid and phenylpropionic acid linkers. J. Pept. Res. 53, 370–382.

Ward, P.D., Tippin, T.K., Thakker, D.R., 2000. Enhancing paracellular permeability by modulating epithelial tight junctions. Pharm. Sci. Technol. Today 3, 346–358.

Wong, V., Gumbiner, B.M., 1997. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J. Cell Biol. 136, 399–409.

Yen, W.-C., Lee, V.H.L., 1994. Paracellular transport of a proteolytically labile pentapeptide across the colonic and other intestinal segments of the albino rabbit: implications for peptide drug design. J. Control. Release 28, 97–109.

Yen, W.-C., Lee, V.H.L., 1995a. Penetration enhancement effect of Pz-peptide, a paracellularly transported peptide, in rabbit intestinal segments and Caco-2 cell monolayers. J. Control. Release 36, 25–37.

Yen, W.-C., Lee, V.H.L., 1995b. Role of Na^+ in the asymmetric paracellular transport of 4-phenylazobenzyloxycarbonyl-L-Pro-L-Leu-Gly-L-Pro-D-Arg across rabbit colonic segments and Caco-2 cell monolayers. J. Pharmacol. Exp. Ther. 275, 114–119.

Zhang, S., Castro, G.A., 1992. Boosted mucosal immune responsiveness in the rat intestine by actively transported hexose. Gastroenterology 103, 1162–1166.