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Reversible Opening of Intercellular Junctions of Intestinal Epithelial and Brain Endothelial Cells With Tight Junction Modulator Peptides

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ABSTRACT

The intercellular junctions restrict the free passage of hydrophilic compounds through the paracellular clefts. Reversible opening of the tight junctions of biological barriers is investigated as one of the ways to increase drug delivery to the systemic circulation or the central nervous system. Six peptides, ADT-6, HAV-6, C-CPE, 7-mer (FDFWITP, PN-78), AT-1002, and PN-159, acting on different integral membrane and linker junctional proteins were tested on Caco-2 intestinal epithelial cell line and a coculture model of the blood–brain barrier. All peptides tested in nontoxic concentrations showed a reversible tight junctions modulating effect and were effective to open the paracellular pathway for the marker molecules fluorescein and albumin. The change in the structure of cell–cell junctions was verified by immunostaining for occludin, claudin-4,-5, ZO-1, β-catenin, and E-cadherin. Expression levels of occludin and claudins were measured in both models. We could demonstrate a selectivity of C-CPE, ADT-6, and HAV-6 peptides for epithelial cells and 7-mer and AT-1002 peptides for brain endothelial cells. PN-159 was the most effective modulator of junctional permeability in both models possibly acting via claudin-1 and -5. Our results indicate that these peptides can be effectively and selectively used as potential pharmaceutical excipients to improve drug delivery across biological barriers.

Introduction

Biological barriers are crucial to protect organisms from harmful agents or pathogens and to establish and maintain the homeostasis of different organs, especially those of the central nervous system.[1,2] However, the same mechanisms of epithelial and endothelial barriers that confer protection also restrict drug absorption and delivery.[1,3] Active efflux transporters not only protect from the harmful effects of xenobiotics but also prevent the penetration of therapeutic drugs.[4] Metabolic enzymes represent another element of the protective system of barriers.[5] In addition, tight intercellular junctions (TJs) limit the free passage of molecules and cells through the intercellular clefts, also called as the paracellular pathway.[1] Because the noninvasive delivery of hydrophilic drugs or biopharmaceuticals to the systemic circulation or to the central nervous system is still a challenge, several methods have been investigated to increase drug delivery across barriers.[3] Absorption enhancers including clinically applied and novel excipients are effective to increase drug permeability across both epithelial and endothelial cell layers[6]; however, these act nonselectively on biological barriers[7] and modulate both the tightness of intercellular junctions and the fluidity of the plasma membrane.[8]

TJs of barrier cells are complex structures composed of integral membrane proteins, linker proteins connecting membrane proteins

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to the actin cytoskeleton, and signaling molecules regulating paracellular tightness and transport.[9] Among the transmembrane TJ proteins occludin and the members of the claudin family have a prominent role in forming paracellular barriers.[10] Adherens junction proteins, such as E-cadherin in epithelial cells or VE-cadherin in endothelial cells, also participate in the regulation of paracellular permeability.[11] Based on the amino acid sequence and/or the structure of these junctional proteins, peptides were designed as novel TJ modulators that directly and specifically interact with the extracellular loops (ECLs).[12]

In our study, 6 different modulator peptides were selected (Table 1) which act at different targets.[1] Two hexameric peptides, ADT-6 and HAV-6, were designed to act on the bulge and groove regions of the first extracellular domain (EC-1) of E-cadherin.[13] These peptides interfere with the homophilic binding of E-cadherin, and time- and dose-dependently increase the paracellular permeability in different epithelial cells.[14,19] Two TJ modulators used in the experiments are derived from microbial toxins. The C-terminal 16-residue long fragment of *Clostridium perfringens* enterotoxin (C-CPE) targets claudins.[15] C-CPE acts specifically at the ECL2 of claudin-4 and -3 to increase intestinal permeability *in vivo* and *in vitro*.[20,21] AT-1002 peptide, an active fragment of zonula occludens toxin, a *Vibrio cholerae* enterotoxin, increases the permeability of epithelial, especially intestinal barriers,[16] similar to the full length microbial pathogenic factor.[22] The last 2 peptides, 7-mer (PN-78) and PN-159, were identified on ethylene glycol tetraacetic acid (a calcium chelating reagent)–treated human bronchial epithelial cells by phage display.[17,18] PN-159 effectively increases the permeability of respiratory epithelium in cultures and in animals.[23] The specific targets of these peptides are unknown; they probably interact with the extracellular domain of integral membrane TJ protein(s). These 6 peptides were not investigated in a comparative way for their efficacy on the same model or for their specificity on different, epithelial versus endothelial barrier models.

For our experiments, 2 well-characterized culture models of the intestinal and the blood-brain barriers (BBBs) were used. The laboratory of Professor Borchardt introduced the Caco-2 cell line as a model system for intestinal epithelial permeability[24] and brain microvessel endothelial cell monolayers as a model to estimate solute permeability through the BBB.[25] Since the pioneering publication on Caco-2 cells, the application of this human intestinal epithelial cell line has become widespread in pharmaceutical research, especially in drug permeability studies.[26] In the field of BBB models, in the last 20 years, monocultures were replaced by coculture models, in which astrocytes and/or pericytes induce BBB characteristics in brain endothelial cells.[27,28] The primary cell–based rat BBB *in vitro* triple coculture model has high complexity and predictive value for drug penetration and was favorably compared with surrogate models of the BBB.[29,30]

TJ modulator peptides hold a great promise as drug absorption enhancers, but more data are needed on their efficacy, mode of action, reversibility, and safety before their application in clinical studies. The expression patterns of tight and adherens junction proteins are specific for different types of barriers providing an opportunity for selective modulation of paracellular permeability, but this approach remained largely unexploited so far. Because of these reasons, the aim of the present study was to compare the efficacy of 6 selected TJ modulator peptides on culture models of both the intestinal barrier and the BBB. In addition to barrier integrity measurements, cell morphology was also investigated. Because safety is a crucial parameter, cellular toxicity and reversibility was also measured. For the selective applications of peptides, the mode of action and/or the target of the TJ modulator is needed; therefore, the potential binding partners of the peptides discovered by phage display were investigated.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich Ltd. (Budapest, Hungary) except for those specifically mentioned.

Peptide Synthesis

The sequence of peptides is shown in Table 1. Peptides were synthesized manually on a 0.5-mM scale with the use of standard Fmoc-chemistry on a Rink-amide resin. Couplings were performed in dimethylformamide with 3-fold excess of N,N′-dicyclohexylcarbodiimide, hydroxybenzotriazole, and Fmoc-amino acids for 3 h at ambient temperature. Fmoc deprotection was performed in 20% piperidine and dimethylformamide mixture for 20 min. The peptides were cleaved from the resin by incubating it with the mixture of trifluoroacetic acid (TFA)/water/triisopropyl silane (48:1:1) at room temperature for 3 h, precipitated and washed with diethyl-ether, and then, lyophilized. Crude peptides were purified using a Shimadzu semipreparative high-performance liquid chromatography system equipped with a Phenomenex Jupiter C18 column in the following solvent system: (A) 0.1% aqueous TFA and (B) 0.1% TFA in 80% aqueous acetonitrile, in a linear gradient mode. Analysis and purity control was carried out on an analytical HPLC instrument (HP Model 1100 liquid chromatograph equipped with a Phenomenex Jupiter C18 column). Quality control of the peptides was done by performing mass spectrometric measurements on a Finnigan TSQ-7000 triple quadrupole mass spectrometer in positive ion mode.

Cell Cultures

Human Caco-2 intestinal epithelial cell line (ATCC cat.no. HTB-37) selected by vinblastine was used until passage 60 for the experiments. Vinblastine treatment (10 nM for 6 passages) induced more homogeneous cell morphology and higher expression level of efflux pumps.[31] Caco-2 cells were grown in DMEM/HAM's F-12 culture medium with stable glutamine (Biochrom GmbH, Berlin, Germany) supplemented with 10% fetal bovine serum (Pan-Biotech GmbH, Aidenbach, Germany) and 50-μg/mL gentamycin in a humidified incubator with 5% CO_2 at 37°C. All plastic surfaces were coated with 0.05% rat tail collagen in sterile distilled water before cell seeding.

Table 1
Characteristics of the Selected Modulator Peptides

Peptide	Target	Mechanism	Amino Acid Sequence	Derived From	Reference
ADT-6	E-cadherin	EC1 domain analogue	Ac-ADTPPV-NH2	Designed to target EC1 domain	13
HAV-6	E-cadherin	EC1 domain analogue	Ac-SHAVSS-NH2	Designed to target EC1 domain	14
C-CPE	Claudin-3, -4	Interact with EC1 domain	NH2-SSYSGNYPYS ILFQKF-OH	Microbial toxin fragment	15
AT-1002	ZO-1	Zot receptor	FCIGRL	Microbial toxin fragment	16
7-mer/PN-78	Unknown	–	FDFWITP	Phage display	17
PN-159	Unknown	–	NH2-KLALKLALKALKLAALKLA-amide	Phage display	18