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Enhancement of Drug Absorption through the Blood Brain Barrier and Inhibition of Intercellular Tight Junction Resealing by E-cadherin Peptides

Paul Kiptoo[†], Ernawati Sinaga^{†,‡}, Anna M. Calcagno[†], Hong Zhao[†], Naoki Kobayashi[†], Usman S.F. Tambunan[‡], and Teruna J. Siahaan^{†,*}

[†]Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, KS 66047, USA

[‡]Department of Chemistry, University of Indonesia, Depok, Indonesia

Abstract

E-cadherin-mediated cell-cell interactions in the *zonula adherens* play an important role in the formation of the intercellular tight junctions found in the blood-brain barrier. However, it is also responsible for the low permeation of drugs into the brain. In this study, HAV6 peptide derived from the EC1 domain of E-cadherin was found to enhance the permeation of ¹⁴C-mannitol and [³H(G)]-daunomycin through the blood brain barrier of the *in situ* rat brain perfusion model. In addition, HAV6 peptide and verapamil have a synergistic effect in enhancing the BBB permeation of daunomycin. A new intercellular-junction resealing assay was also developed using Caco-2 monolayers to evaluate new peptides (BLG2, BLG3, and BLG4) derived from the bulge regions of the EC2, EC3, and EC4 domains of E-cadherin. BLG2 and BLG4 peptides but not BLG3 peptides were found to be effective in blocking the resealing of the intercellular junctions. The positive control peptides (ADT10, ADT6, and HAV10) block the resealing of the intercellular junctions in a concentration-dependent manner. All these findings suggest that E-cadherin-derived peptides can block E-cadherin-mediated cell-cell interactions. These findings demonstrate that cadherin peptides may offer a useful targeted permeation enhancement of therapeutic agents such as anticancer drugs into the brain.

Keywords

E-cadherin; cell-cell adhesion; HAV peptides; ADT peptides; intercellular junctions; adherens junction; Caco-2 cell monolayers

Many drug molecules have difficulty in crossing the intestinal mucosa and the blood-brain barrier (BBB).^{1, 2} For proteins and peptides, their size and hydrophilicity prevent them from crossing these biological barriers. They cannot penetrate the paracellular pathway due to the presence of intercellular tight junctions.³ Intercellular tight junctions are circumferential zipper-like seals between adjacent endothelial cells of the BBB. The tight junctions have multiple functions, including maintaining cell polarity to prevent the mixing of membrane proteins between the apical and basolateral membranes⁴ and functioning as a gate to control the paracellular passage of ions and solutes.⁵ The adherens junctions consist of major transmembrane proteins called classical cadherins (i.e., E-cadherin), which generate intercellular contacts through trans-pairing between cadherins on opposing cells.^{6, 7} The

*To whom correspondence should be addressed Dr. Teruna J. Siahaan, Department of Pharmaceutical Chemistry, The University of Kansas, 2095 Constant Ave., Lawrence, KS 66047, Phone: 785-864-7327, Fax: 785-864-5736, siahaan@ku.edu.

tight junctions of BBB begin to restrict the diffusion of molecules with molecular weight higher than 180 Da and there is a relationship between molecular size and brain absorption.⁵ Although small hydrophobic anticancer agents can readily partition into cellular membranes, the drug permeation through the BBB can be limited by the presence of ABC efflux pumps (*i.e.*, P-glycoprotein (Pgp) and multidrug resistance-associated proteins (MRPs) as well as extensive Phase-I and -II metabolisms.^{8, 9}

One way to improve the delivery of both large hydrophilic molecules and small anticancer agents into the brain is by enhancing their permeation through the paracellular pathways of the BBB. In this case, modulation of the cadherin-cadherin interactions in the adherens junction could increase the porosity of the intercellular tight junctions. It has been shown that the opening and resealing of tight junctions can be controlled upon removing and restoring extracellular calcium ions.^{10, 11} His-Ala-Val (HAV) and Ala-Asp-Thr (ADT) peptides derived from the EC1 domain of E-cadherin can modulate the porosity of the intercellular junctions of Madin-Darby Canine Kidney cell monolayers (MDCK); these peptides lower the transepithelial electrical resistance (TEER) and increase paracellular permeation of ¹⁴C-mannitol through MDCK cell monolayers.^{12, 13} In the absence of reproducible and tight model of the BBB, Caco-2, and MDCK cell monolayers can only be used to screen molecules that can penetrate the biological barriers. However, more studies are needed to design peptide analogues that can specifically modulate the intercellular junction of BBB and to minimize systemic interference due to peptide cross-reactivity with various cadherins on tissues other than the BBB. Binding specificity of classical type-I cadherin has been extensively shown to be primarily homophilic.^{14, 15} For example, cells expressing N-cadherin preferentially adhere to other cells expressing N-cadherin but not to cells expressing E-cadherin. It was proposed that the N-terminal region of cadherin (extracellular domain-1 or EC1) provides selectivity of cadherin. For example, L cells expressing PE386, PE229, and PE113 that contained amino-terminal P-cadherin sequences could not aggregate with L cells expressing the wild-type E-cadherin ELs.¹⁶ However, several recent studies have indicated that interaction between cadherins can be more promiscuous than previously suggested.^{17–19} Point mutation analyses suggest that binding specificity of cadherin is determined by multiple sites located at the N-terminal extracellular domain-1 (EC1 domain).¹⁶ A comparison of the EC1 domain of various cadherins shows that this region is highly conserved with 65% identity (74/113 amino acids) between E- and P-cadherins. Thus, the specificity of each cadherin is determined by a relatively small number of non-conserved amino acids.¹⁶ It is important to note that the His-Ala-Val (HAV) sequence is found in type-I classical cadherins, including E-, N-, P-, VE-, and R-cadherins. We propose that to confer specificity to a particular cadherin, it might be necessary to invoke a specific conformation of the HAV peptides by forming cyclic peptides. Thus, we are currently investigating the effect of cyclization of HAV peptides to provide selectivity for particular cadherins that are expressed in the BBB.

Our future goal is to enhance the transport of larger molecules (*i.e.*, protein therapeutics) into the brain to treat brain diseases. However, before we can accomplish this goal, the concept of using the HAV peptide to enhance permeation of small molecules (*i.e.*, mannitol and daunomycin) should be proven viable. In this study, HAV6 peptide was used to modulate the BBB in *in situ* rat brain perfusion and the effect was determined by the enhancement of brain delivery of ¹⁴C-mannitol and [³H(G)]-daunomycin. Next, a resealing assay of Caco-2 cell monolayer was developed to evaluate the efficacy of cadherin peptides in blocking the homophilic interactions of E-cadherins. The feasibility of using the resealing assay to evaluate the activity of E-cadherin peptides was determined using positive control peptides such as HAV10, ADT10 (BLG1), and ADT6. This assay also was used to evaluate new BLG2, BLG3, and BLG4 peptides derived from the bulge regions of the EC2, EC3, and EC4 domains of E-cadherin, respectively. The potential mechanism of action of these

peptides was evaluated by blocking E-cadherin-mediated homotypic single cell adhesion to modified Caco-2 cell monolayers as well as by inhibiting calcium-dependent re-aggregation of single Caco-2 cells.

MATERIALS AND METHODS

Materials

All components for the cell culture medium, Hank's balanced salt solution (HBSS) with and without CaCl_2 (approximately 1.26 mM), and ($\pm$)-verapamil were purchased from Gibco (Carlsbad, CA) or Sigma (St. Louis, MO). [$^3\text{H}(\text{G})$]-daunomycin (19.7 Ci/mmol) and ^{14}C -mannitol were purchased from PerkinElmer Inc. (Boston, MA). Ten to 12-wk-old male Sprague-Dawley rats weighing 300–400 g were purchased from Charles River (Wilmington, MA) and housed under specific pathogen-free conditions in the animal facility at the University of Kansas, which is approved by the Association for Assessment and Accreditation of Laboratory Animal Care. All protocols involving rats were approved by the Institutional Animal Care and Use Committee of the University of Kansas.

Peptide synthesis

Cadherin peptides (Table 1) were synthesized using solid-phase synthesis with Fmoc-chemistry.^{12, 13} Peptides were cleaved from the resin using standard methods and purified by semi-preparative reversed-phase HPLC using a C_{18} column. All peptides were shown to have >95% purity by analytical HPLC; their identities were confirmed by mass spectrometry.

In situ rat brain perfusion technique

The permeation of ^{14}C -mannitol and [$^3\text{H}(\text{G})$]-daunomycin into the brain following modulation of the BBB by HAV6 peptide was evaluated using the *in situ* rat brain perfusion technique developed by Takasato et al. (1984) with slight modification.²⁰ Prior to surgery, the animals were anesthetized with intraperitoneally administered ketamine (100 mg/kg) and xylazine (5 mg/kg). The pterygopalatine, occipital, and superior thyroid arteries were coagulated and cut, and the left common carotid artery (LCCA) was prepared for ligation by loosely encircling the artery with surgical silk just proximal to the bifurcation of the external carotid artery. A polyethylene catheter (PE-50) filled with heparinized saline (100 IU/mL) was then placed in the LCCA for retrograde perfusion. The rectal temperature was maintained at $36.5 \pm 0.5^\circ\text{C}$ throughout the experiment by a heat lamp connected to a feedback device (YSI model 73 ATD indicating controller). Before perfusion, the perfusate was filtered, oxygenated with a mixture of 95% air and 5% CO_2 , and heated to 37°C in a water bath.

For ^{14}C -mannitol, the perfusate consisted of 1.0 or 0.5 mM test peptides (HAV6, LABL6) in vehicle (0.5 % Tween in sterile saline). Perfusion by an infusion pump (Saga Instruments, Cambridge, MA, model 355 syringe pump) at 10 ml/min through the LCCA was started immediately after a heart-cut on the anesthetized rat and ligation of the LCCA. The perfusion protocol consisted of a 20-s pre-perfusion wash (saline only), a 240-sec perfusion (vehicle containing test compounds), a 240-sec perfusion of ^{14}C -mannitol in sterile saline, and a 5-sec post-perfusion wash (saline only) controlled by a switching valve. The perfusion was terminated by decapitation of the animal.

For the delivery of [$^3\text{H}(\text{G})$]-daunomycin, immediately after performing a heart-cut on the anesthetized rat and ligating the left common carotid artery, the peptide solution (1.0 mM HAV6 in sterile saline with 0.5% Tween-20) was perfused using an infusion pump at a flow rate of 5.0 ml/min for 4 min into the left hemisphere of the rat brain via a syringe connected

to the catheter in the left external carotid artery. Following infusion of the peptide, [$^3\text{H}(\text{G})$]-daunomycin (15 μCi with or without verapamil (500 μM) in sterile saline with 0.5% Tween-20) was immediately perfused into the rat brain as described above. After a period of 8 min, the perfusion was terminated by washing the catheters with sterile saline at a flow rate of 5.0 ml/min for 10 s followed by decapitation of the animal. The rat brain was removed from the skull and dissected on ice. All parts of the brain tissue sample (50–60 mg) were weighed, ground with a mortar and pestle, dissolved in scintillation fluid, and counted for radioactivity using a dual-label scintillation spectrometer (Beckman LS 6000 IC). Each experiment was performed in triplicate.

Cell Culture

The colon adenocarcinoma-2 (Caco-2) cell line was purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown in 75 cm^2 tissue culture-treated polystyrene flasks (Fisher Scientific, Pittsburgh, PA) in a controlled atmosphere of 5% CO_2 and 95% relative humidity at 37°C. Culture medium consisted of 13.48 g/l Dulbecco's modified Eagle medium (DMEM) supplemented with 3.7 g/l sodium bicarbonate, 1.42 g/l Hepes sodium, 0.1 mM non-essential amino acids (NEAA), 2.0 mM glutamine, 10% fetal bovine serum, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Cell culture medium was replaced every other day for the first six days and then daily thereafter for Caco-2 cells. Cell monolayers at 80–90% confluency were trypsinized with 0.25% trypsin in 1.0 mM EDTA. One-fifth of the cell suspension was subcultured every 5–7 days. For re-aggregation studies, the cells were seeded on 24-well plates (Fisher Scientific).

Cytotoxicity Assay

The cytotoxicity of the HAV derivatives was evaluated using an MTT assay. Briefly, Caco-2 monolayers were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a 5% CO_2 incubator. Cells were seeded in 24-well plates in 2 mL medium per well at a density of 10,000 cells/well for 6 h. The medium was then replaced with 2 mL of medium containing indicated concentrations of HAV peptides as well as various controls and incubated for 24 h under 5% CO_2 at 37°C. After 24 h, the culture medium was removed from the cell monolayers and cultured in 96-well plates. Each well was reconstituted with 0.2 mL supplemented RPMI-1640 containing 1 mg/mL MTT and incubated for 2–4 hrs at 37°C. The supernatants were removed from the wells and then 0.2 mL dimethyl sulfoxide (DMSO) per well was added in isopropanol and mixed with the contents of the wells thoroughly. Finally, the plates were read in an automated microplate spectrophotometer (ELX800 Biotek, USA) at 570 nm as a reference. Cell viability was determined by the following formula:

$$\text{Cell viability (\%)} = \frac{OD(\text{test well})}{OD(\text{reference well})} \times 100\%$$

Inhibition of Intercellular Junction Resealing in Caco-2 Monolayers

Caco-2 cells were seeded on polystyrene filter inserts (3 μm pore) inside 12-well Transwell™ plates (Costar, Cambridge, MA) at a density of 50,000 cells per Transwell™. Experiments were performed using Caco-2 monolayers on days 15–20.

The modulation of intercellular junctions within cell monolayers by cadherin peptides and controls (i.e., using no peptide or control peptides) was evaluated by observing the change in TEER values during the resealing of the intercellular junctions measured by Evom Epithelial Voltometer (World Precision Instruments, Inc., Sarasota, FL). Briefly, the cell monolayers were washed with pH 7.4 Hank's balanced salt solution (HBSS) containing 10 mM Hepes,

1% glucose, and 2.0 mM CaCl_2 . Initial TEER values were determined after the monolayers were incubated in HBSS (pH 7.4) with CaCl_2 at 37 °C for 1 h for Caco-2 cells. Conditioning was conducted by incubating the cell monolayers in calcium-free medium for 2 h prior to treatment with peptide solution containing CaCl_2 . Then, 1.0 mM peptide solutions in HBSS (pH 7.4) with CaCl_2 were added to the apical (AP), basolateral (BL), or both sides (AP-BL) of the Transwell™. TEER values were measured every hour. For controls, HBSS with CaCl_2 without peptide (blank) or with 1.0 mM unrelated control peptide was applied to both sides concurrently. TEER values were measured for an additional 5–7 h at 1 h intervals. Background resistance was determined as $90 \Omega \times \text{cm}^2$ in wells that were unseeded and incubated with HBSS. This value was subtracted from each measurement. The experiments were performed in triplicate.

Re-aggregation studies

To evaluate the activity of cadherin peptides in inhibiting single cell re-aggregation, cell monolayers grown in 24-well plates were washed 3 times with Ca^{2+} - and Mg^{2+} -free HBSS (pH 7.4) followed by incubation for 3 h in the same solution. This treatment disrupts the intercellular junctions to release single cells from the monolayers. Then, re-aggregation of single cells was induced by incubation with HBSS containing 2.0 mM CaCl_2 in the absence or presence of 1.0 mM peptide. The cell re-aggregation was observed under a phase-contrast microscope every hour up to 5 h of incubation with this calcium-containing medium. Pictures were taken at several time points.

Homotypic adhesion of Caco-2 single cells to attached cells

BLG1, BLG2, BLG3, and BLG4 peptides were used to block E-cadherin-mediated cell adhesion by inhibiting adhesion of Caco-2 single cells to modified Caco-2 cell monolayers. Briefly, single Caco-2 cells were derived from cell monolayers that were treated with Ca^{2+} - and Mg^{2+} -free Earle's balanced salt solution (EBSS) for 1 h at 37 °C. The single cells were incubated with 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, Invitrogen, Carlsbad, CA). Then, the BCECF-labeled single cells (5×10^4 cells/well) were added to the modified Caco-2 cell monolayers in 48-well plates. The modified cell monolayers had been pretreated with Ca^{2+} -free EBSS (1 mM Mg^{2+} , 0.1% BSA) for 2 h at 37°C to expose the E-cadherin to the cell surface from the adheren junctions. This cell mixture was incubated for 2 h at 37°C in the presence or absence of various concentrations of peptides. As a positive control, a 1:100 dilution of anti-E-cadherin antibody (clone DECMA-1, Sigma-Aldrich) was used. After removing the unbound cells, the attached cells were lysed in lysis buffer and the fluorescence intensity of each sample was measured using a microplate fluorescence analyzer (Bio-Tek FL600) at λ_{ex} 485 nm and λ_{em} 530 nm.

RESULTS

Enhancement of ^{14}C -mannitol and $^3\text{H}(\text{G})$ -daunomycin permeation across the in vivo BBB

The ability of HAV6 peptide to modulate the intercellular tight junctions of the BBB was evaluated by measuring the enhancement of permeation of a paracellular marker molecule (i.e., ^{14}C -mannitol) through the BBB in an *in situ* perfused rat brain (Fig. 1). Here, *in situ* perfusion was conducted as described by Liu et al. (2004) in their investigation with PBS as perfusate with a flow rate of 10 mL/min.²² In earlier studies, the perfusion flow rates were determined using regional blood flow rates. The regional flow rate in the cortex was determined using the BBB permeability-surface area (PS, quantified as logPS) value of diazepam. Diazepam is a highly permeable compound that has been used as a regional flow rate marker in brain perfusion studies.²⁰ Using diazepam, the measured regional flow rate was 0.070 ± 0.022 mL/s/g at a 10 mL/min perfusion rate. In our studies, gross inspection of

the rat brains isolated after completion of the perfusion did not reveal any signs of edema, suggesting that no membrane damage occurred at this flow rate. It should be noted that a high hydrostatic pressure could compromise the integrity of tight junction especially when dealing with other perfusate such as whole blood at a high flow rate during perfusion. Takasato et al. (1984) showed that vascular resistance was significantly greater with perfusion of whole blood (1550 ± 177 mmHg.s.mL⁻¹) or of artificial blood (1200 ± 117 mmHg.s.mL⁻¹) compared to perfusion with H₂CO₃ saline (900 ± 83 mmHg.s.mL⁻¹).²⁰ Pretreatment of rat brain with 1.0 mM HAV6 peptide significantly enhanced the disposition of ¹⁴C-mannitol (11425.3 ± 3983.6 cpm) in the brain compared to vehicle (0.5% Tween-20 in sterile saline; 3148.1 ± 1311.7 cpm). Treatment with 1.0 mM LABL6 (control peptide) did not enhance the paracellular penetration of ¹⁴C-mannitol through the BBB. The amount of mannitol delivered to the brain was 40% lower when the concentration of HAV6 was lowered from 1.0 mM to 0.5 mM, suggesting that the activity of HAV6 was concentration-dependent. A known tight junction modulator called palmitoyl carnitine (1.0 mM) was used as a positive control; it enhanced the transport of ¹⁴C-mannitol similar to treatment with 1.0 mM HAV6 (i.e., no significant difference; $p > 0.05$). Palmitoyl carnitine has been shown previously to modulate tight junctions of the intestinal mucosa. It increases intestinal mucosa transport of hydrophilic molecules²³ and water transport across rat corneal endothelium.²⁴ Although HAV6 peptide modulates the BBB at relatively high concentration, it is exciting to find that this is the first proof-of-concept result to show HAV peptide can enhance the permeation of drug molecule through the BBB in *in vivo* system. The HAV6 peptide and its derivatives were not toxic up to 4 mM of peptides in Caco-2 cells (Figure 2). In the future, the concentration can be lowered by designing cyclic HAV peptides and mutating the amino acids to improve peptide-binding affinity and selectivity to a particular cadherin.

The activity of HAV6 peptide in increasing the BBB penetration of [³H(G)]-daunomycin was also evaluated. In this study, the rat brain was first perfused with solutions containing 1.0 mM HAV6, 1.0 mM HAVscr (scrambled peptide), and vehicle only (no peptide). Then, a solution of [³H(G)]-daunomycin (15 μ Ci) was delivered, followed by evaluation of radioactivity in the brain. It is interesting to find that the amount of [³H(G)]-daunomycin was significantly higher ($p < 0.05$) in rat brains perfused initially with HAV6 (Figure 3, C) compared to those treated with vehicle (Figure 3, A). Another negative control, the scrambled HAV peptide (HAVscr), did not enhance the permeation of [³H(G)]-daunomycin into the brain (Figure 3, B); there was no significant difference ($p > 0.05$) compared to those treated with vehicle.

In addition to the physical barrier, permeation of daunomycin into the brain is also limited by the activity of P-glycoprotein (P-gp) expressed in the brain capillary endothelium.⁹ Thus, the effect of HAV6 in the absence and presence of an efflux pump inhibitor verapamil was evaluated. First, the rat brain was perfused with vehicle followed by a perfusate containing 15 μ Ci [³H(G)]-daunomycin and 500 μ M verapamil. In this experiment, verapamil significantly enhanced the brain transport of daunomycin ($p < 0.05$) compared that of vehicle-treated BBB (Figure 3, D). Second, the BBB was treated first with 1.0 mM HAV6 and then immediately followed by a mixture of daunomycin and 500 μ M verapamil. The result showed a tremendous increase in the amount of daunomycin in the brain compared to vehicle-treated BBB ($p < 0.001$) (Figure 3, E). The combination treatment (HAV6 + verapamil) was better than HAV6 or verapamil alone. Finally, the BBB was treated with 1.0 mM HAV6 and 500 μ M verapamil followed by perfusion of 15 μ Ci [³H(G)]-daunomycin and 500 μ M verapamil. The highest cumulative amount of [³H(G)]-daunomycin was observed in the brain (Figure 3, F) with this treatment method; the amount of daunomycin (18192 ± 3358 cpm) was five-fold that of treated vehicle (3388 ± 884 cpm).

The effect of HAV peptide on the length of time of disruption of the BBB was not evaluated; therefore, it is difficult to predict how long the tight junction stays open without conducting elaborate experiments. It is possible that this disruption could be much longer *in vivo* than *in vitro* because the *in vivo* BBB is more dynamic than the *in vitro* cell culture model. More experiments are needed in future to evaluate the duration of intercellular junction opening in the BBB. In parallel, the pore-size opening caused by the peptide will be determined.

Using *in vitro* assay in MDCK monolayer, modulation of the intercellular junction by the HAV peptide (Ac-SHAVSS-NH₂) showed some minimal return of TEER values after 6 h incubation, although the return was not completely back to 100% of the original TEER value.¹² The less potent and less stable derivatives of HAV peptides showed the junction resealing back to 100% after 6 h incubation; the return may be due to the degradation of the peptides. The HAV peptide stability was evaluated by incubating the peptide on the apical side of Caco-2 monolayers at 10 and 100 μ M concentrations. At 100 μ M concentration, the HAV10 peptide concentration reduces to 80% upon incubation for 8 h at apical side of Caco-2 cell monolayers. In the future, we will study the stability of these peptides in plasma. Also, we will design *in situ* perfusion experiments using whole blood as the vehicle.

Inhibition of Intercellular Junction Resealing by Cadherin Peptides in Caco-2 Cell Monolayers

To expand the possibility of searching for new peptides that can modulate E-cadherin-mediated intercellular junction adhesion, the intercellular junction-resealing assay was evaluated in Caco-2 cell monolayers. In this study, the integrity of the intercellular junctions of the cell monolayer was disrupted by incubation with calcium-deficient medium for 2 h followed by intercellular junction resealing upon switching from calcium-deficient to calcium-sufficient medium in the presence and absence of peptides (i.e., cadherin peptides or controls). The TEER values were measured every hour for 6 h to monitor the resealing of the intercellular junctions. The monolayers treated with VVA peptide had normal TEER values after 6 h (8-h time point), suggesting that the control peptide could not block the resealing of the intercellular junctions. In contrast, HAV10 peptide could inhibit the resealing of intercellular junctions at 1.0 mM but not at 0.5 mM. In addition, the TEER values could not return to 100% at the 8-h time point upon peptide inhibition (Figure 4A). Similarly, ADT10 peptide inhibited the resealing of the intercellular junctions in a concentration-dependent manner; unlike HAV10, ADT10 was still active at 0.5 mM concentration (Figure 4B). A smaller ADT6 hexapeptide also inhibited the resealing of the intercellular junctions in concentration-dependent manner (Figure 4C), suggesting that ADT6 still maintains the recognition sequence for inhibition of intercellular junction resealing and may have better activity than HAV10.

Due to structural-motif similarity of EC domains (EC1 through EC5), peptides (BLG2, BLG3, BLG4, and BLG5) from the bulge region of EC2, EC3, EC4, and EC5, respectively, may have inhibitory properties similar to those of ADT10 (or BLG1) from the EC1 domain. Thus, BLG2, BLG3, and BLG4 were evaluated in the resealing assay; due to difficulty with its synthesis and handling, BLG5 from EC5 was not evaluated. To compare the activities of all the peptides, the % TEER difference between 8- and 2-h time points was plotted for each compound. Thus, the better the peptide is in blocking the resealing, the lower the percent change (Figure 5); these data were from peptide addition to AP-BL sites of the monolayers. The results show that BLG1 (ADT10), BLG2, and BLG4 significantly suppress the resealing of the intercellular junctions compared to blank control. BLG4 showed the best inhibitory activity. In contrast, BLG3 could not inhibit the resealing of the intercellular junctions. The control scrambled HAVscr and reversed HAVrev peptides could not suppress

the resealing of the junctions. These results suggest that peptides from bulge regions other than those from EC1 can be used to modulate the in vivo BBB.

Inhibition of Caco-2 Single Cell Adhesion to Attached Cells

The proposed mechanism of inhibition of intercellular junction resealing by the peptides (BLG1, BLG2, and BLG4) is assumed to be via inhibition of E-cadherin-mediated cell-cell adhesion. To evaluate this likelihood, these peptides were used to inhibit calcium-dependent Caco-2 single cell adhesion to attached Caco-2 cells. This homotypic adhesion can be inhibited by anti-E-cadherin antibody (Fig. 6), suggesting that this process is mediated by E-cadherin.²¹ It was interesting to find that BLG1 (ADT10), BLG2, and BLG4 peptides significantly inhibited cell adhesion in a concentration-dependent manner, suggesting that they block the cell adhesion by binding to E-cadherin. At 250 μ M concentration, BLG2 was more potent than the same concentration of BLG1 and BLG4 in blocking homotypic cell-cell adhesion. It should be noted that HAV10 peptide had been shown previously to inhibit the homotypic Caco-2 cell adhesion.²¹

Inhibition of Single Caco-2 Cell Re-aggregation

Another method of evaluating the effect of peptides in inhibiting E-cadherin-mediated cell-cell adhesion is by inhibiting Caco-2 cell re-aggregation. Here, Caco-2 monolayers were treated with calcium-free medium for 3 h to loosen the cell-cell adhesion in the monolayers to give single cells. Then, the medium was replaced with HBSS containing 2.0 mM CaCl_2 in the absence and presence of 1.0 mM peptides (BLG1 (ADT10), HAV10 and HAV6) for 5 h. In the absence of peptide, nearly all cells re-aggregated during the 1-h recovery period in the presence of CaCl_2 (Figure 7A). On the other hand, the re-aggregation of Caco-2 cells was inhibited by the peptides in a time-dependent manner (Figures 7B and C). The dissociated cells were more pronounced after 5-h incubation with the BLG1 peptide (Figure 7C) than after 1-h incubation (Figure 7B). In other words, fewer cell aggregates were observed after 5-h incubation than at the 1-h time point. Similarly, HAV10 and HAV6 peptides also inhibited the re-aggregation of the Caco-2 cells. These results indicate that, upon binding to E-cadherin, these peptides can block the E-cadherin-mediated cell re-aggregation process.

DISCUSSION

There is a major problem in delivering medium and large hydrophilic molecules such as peptides and proteins through the BBB and intestinal mucosa barriers. Similarly, some lipophilic anticancer drugs may not penetrate the BBB due to their recognition by the efflux pumps. Thus, if there is a way to improve the delivery of these molecules to the brain, many therapeutic agents that previously could not be used to treat the central nervous system (CNS) could be reevaluated for their use in the CNS. Although cadherin peptides (i.e., HAV6, HAV10, ADT10, and ADT6) have previously been shown to enhance ^{14}C -mannitol through MDCK cell monolayers,^{12, 13} this is the first evidence to show that a cadherin peptide such as HAV6 peptide can enhance the permeation of mannitol and daunomycin through the BBB in rats. This enhancement of brain disposition of mannitol and daunomycin could be due to the increase in porosity of the tight intercellular junctions of the BBB created by HAV6 peptide. Previously, tight-junction modulators such as EDTA, palmitoyl-DL-carnitine, and deoxy- or glycol-cholate or chitosan have been shown to significantly enhance the permeation of low molecular weight and polar solutes (i.e., mannitol, urea, lactate, or atenolol) through biological barriers.^{25–28} Unfortunately, some of these enhancers open the BBB via non-selective mechanisms. For example, EDTA opens the tight junctions by depleting calcium ions; calcium depletion has been shown to also open the intercellular junctions of cell monolayers (i.e., Caco-2 and MDCK).

Due to its hydrophobic nature, daunomycin normally penetrates the BBB via the transcellular route; however, due to the presence of Pgp on the brain vascular endothelial cells, its brain transport is limited. Previously, a Pgp inhibitor, verapamil, has been shown to enhance the BBB permeation of D-phenylalanine-containing peptides by saturating the efflux transport mechanism.²⁹ As expected, verapamil increases the transport of [³H(G)]-daunomycin significantly into the brain compared to untreated control; verapamil causes improvement in the transcellular transport of daunomycin (Figure 3, D). Without verapamil, HAV6 peptide enhances the permeation of [³H(G)]-daunomycin into the brain at a level similar to that of verapamil (Figure 3, C). In contrast, the scrambled peptide (HAVscr) did not enhance the transport of daunomycin, suggesting that the activity of HAV6 peptide is sequence-specific. We and others have confirmed the importance of the HAV sequence in cadherin.^{12, 13, 30-35} In the absence of verapamil, HAV6 peptide opens the intercellular junctions to allow the majority of daunomycin to permeate the BBB via the paracellular route rather than the transcellular route. Although it is less likely, HAV6 peptide could also inhibit Pgp to allow the transcellular permeation of daunomycin. It is interesting to find that HAV6 peptide and verapamil have a synergistic effect in enhancing brain delivery of daunomycin up to fivefold compared to control. Although there is no evidence that HAV peptides can influence the function of Pgp, our data suggest that HAV peptide modulation does not influence the Pgp properties because the HAV peptide and verapamil combination appear to improve the transport of daunomycin in an additive manner. Therefore, the two enhancers have different mechanisms of action. In the future, it will be valuable to determine the component of daunomycin that penetrates the BBB through the paracellular or transcellular route upon treatment with various ratios of HAV6 peptide and verapamil.

ADT10 (BLG1) and ADT6 peptide from the bulge region of EC1 domain of E-cadherin can modulate the intercellular junctions of MDCK cell monolayers and enhance the permeation of ¹⁴C-mannitol in vitro.¹³ Thus, other peptides from the bulge region of other domains could also modulate the intercellular junctions. In this case, BLG2, BLG3, and BLG4 peptides were synthesized from the bulge region of EC2, EC3, and EC5 and evaluated in the Caco-2 resealing assay. The advantage of the resealing assay over the regular monolayer is that the resealing assay can be used to evaluate the activity of medium and large inhibitors of cadherin-cadherin interactions (i.e., peptides and proteins). The large inhibitor can reach the target cadherins deep in the adherens junctions for initial evaluation. ADT10 (BLG1), ADT6, and HAV10 were used as positive control molecules to validate the viability of the resealing assay, and these peptides were effective in inhibiting the resealing of the intercellular junctions of Caco-2 cell monolayers in a concentration-dependent manner (Fig. 4). It is interesting to find that BLG2 and BLG4 from the bulge regions of EC2 and EC4 domains, respectively, blocked the resealing of intercellular junctions of Caco-2 monolayers (Fig. 5), but the BLG3 peptide from the EC3 domain could not. There are several potential explanations for the inactivity of BLG3 peptide. First, BLG3 does not bind or bind with low affinity to E-cadherin on the cell surface. Second, BLG3 may bind to E-cadherin but not in the cadherin-cadherin binding region. Third, the EC3 domain may not play a critical role in maintaining tight junctions; thus, the peptides from this domain may not have any effect on modulating E-cadherin interactions. As a second confirmation, BLG1, BLG2, and BLG4 can also inhibit calcium-mediated Caco-2 single cell adhesion to modified monolayers in a concentration-dependent manner, indicating that these peptides inhibit the homotypic cell-cell adhesion by binding to E-cadherins (Fig. 6).

To test the possible synergistic effect of HAV and ADT peptides, ADT6 and HAV6 from the bulge and groove regions of the EC1 domain were conjugated via aminocaproic acid (cap) linker to give Ac-DTPPV-cap-SHAVSS-NH₂ and Ac-SHAVSS-cap-ADTPPV-NH₂ conjugates. These peptides could inhibit the resealing of the intercellular tight junctions but they did not show any improved activity when compared to either ADT6 or HAV6,

suggesting that there was no synergistic effect of the two peptides. The lack of improvement in activity may be due to (a) non-optimal distance between the two peptides so they can function synergistically and (b) two peptides from a folded structure that may mask the active sequences to lower its activity. Unfortunately, the synergistic effect of a mixture of ADT6 and HAV6 peptides has not been evaluated.

The presence of tight junctions is one of the impediments in the penetration of therapeutic molecules through biological barriers to reach their target sites of action. It has been suggested that the formation of tight junctions occurs in response to the primary interaction of cell adhesion molecules such as cadherin.³¹⁻³⁶ Modulation of integrity of the intercellular junctions by cadherin peptides depends on the cell type. We found Madin-Darby Canine Kidney (MDCK) cell monolayers more sensitive to modulation by cadherin peptides than Caco-2 cell monolayers. The TEER reduction of MDCK monolayers was faster than that of Caco-2 monolayers during the calcium depletion process. The more selective effect of HAV6 peptide to MDCK monolayers compared to Caco-2 cell monolayers may not be due to the more rapid metabolism of the peptide in Caco-2 monolayer because HAV6 peptide is metabolically stable upon 96-h incubation on Caco-2 cell monolayer. One possible explanation for the selective effect of HAV6 is that the structure and tightness of the intercellular junctions of Caco-2 cell monolayer are different from the MDCK monolayer. The second potential explanation is that the porosity of the tight junctions in Caco-2 monolayer is smaller than MDCK cell monolayer; thus, the lower porosity prevents the HAV6 peptide from reaching E-cadherins in the adheren junctions. Finally, the expression and the interaction between cadherins could be tighter in Caco-2 than in MDCK cell monolayers.

In the absence of reliable cell culture model for the BBB with tight intercellular junctions, the MDCK cells transfected with human MDR1 gene has been used as surrogate model to rapidly screen the BBB transport properties of potential drugs.³⁷ However, the results from this study should be interpreted with caution because MDCK cells are epithelial cell but not endothelial. In addition, these cells are derived from kidney and not from brain capillaries. Therefore, it is necessary to evaluate the drug transport using the *in vivo* or *in situ* models to confirm the findings from screening using MDCK cell monolayers.

Previously, calcium-mediated cell-cell aggregation has been used to evaluate E-cadherin-mediated cell-cell adhesion.³⁸ Here, cadherin peptides were shown to inhibit calcium-mediated Caco-2 cell re-aggregation in a time-dependent manner (Fig. 7); fewer cell aggregations were found upon 5-h incubation with the peptides than after 1-h incubation. Thus, it is proposed that cadherin peptides increase the porosity of the intercellular junctions of BBB or cell culture monolayers by blocking the cadherin-cadherin interactions. NMR spectroscopy has indicated that the HAV6 and BLG4 peptides bind the expressed EC5 domain, supporting a proposal that these peptides inhibit cadherin-mediated cell-cell adhesion in the intercellular junctions by binding to cadherins.³⁹ In future, the binding properties of HAV6 and BLG4 peptides to EC1, EC2, EC3, or EC4 domains of E-cadherin will be evaluated to determine whether the activity of cadherin peptides is due to their binding to multiple domains of E-cadherin.

Calcium is necessary to rigidify E-cadherin for mediating cell-cell adhesion to establish the intercellular junctions, and removal of calcium induces the dissociation of cadherin-cadherin interaction.⁴⁰⁻⁴¹ However, it is difficult to pin point how extracellular calcium depletion disrupts the intercellular junction integrity because a decrease in extracellular calcium concentrations also causes a variety of changes within the cells. Calcium depletion causes rapid changes in MDCK cell shape as seen by increases in cell height and decreases in cell area without cell detachment from the substratum.⁴²⁻⁴⁴ Calcium levels and protein kinase

activity play a vital role in both the structure and function of the tight junctions.^{45–47} Low extracellular levels of calcium trigger signal transduction pathways that involve protein kinases and cause the removal of various junctional proteins.⁴⁵ In keratinocytes, protein kinase C activation in low calcium concentrations caused the reorganization of E-cadherin-mediated adherens junctions.⁴⁸ The reassembly of tight junctions following calcium depletion involves E-cadherin-regulated adhesion as the first step,⁴⁹ followed by ZO-1 localization at the cell surface prior to the localization of 25 emophili. In addition, ZO-1 accumulates at the adherens junction with claudin, and claudin is then transported to the apical tight junction.

Depletion of extracellular calcium also causes an influx in sodium ions, which appears to either release calcium from intercellular storages or prevent the uptake of calcium to those storages.⁴⁴ In studies in which calcium switching was performed from low (<5 μ M) to high (1.8 mM) levels of extracellular calcium, intracellular calcium levels increased without a significant increase in intracellular pH.⁵⁰ The extracellular calcium depletion can move the actin network toward the center of the cell and cause the contraction of muscle cells.^{42, 44} There are also contradictory reports about the integrity of the actin ring following calcium depletion,^{43, 51} and intercellular calcium chelation has been shown to have no effect on E-cadherin or the reorganization of the actin cytoskeleton.⁵² Because calcium ions affect many cellular processes and proteins, it is difficult to single out a mechanism of tight junction reorganization during calcium depletion. We also have shown that there is a redistribution of E-cadherin from the junction to the surface of the cells during the calcium depletion process. Following incubation with calcium for 3 h, however, the E-cadherin was found primarily in the intercellular junctions.

In conclusion, this study provides a proof-of-concept that cadherin peptides can be utilized to improve the delivery of drug molecules into the brain by modulating the paracellular pathways of the BBB. HAV6 peptide in combination with verapamil can improve penetration of the anticancer drug daunomycin into the brain. In addition to previously studied cadherin peptides (i.e., ADT10, ADT6, HAV10, HAV6), two new peptides (BLG2 and BLG4) were found to be active in inhibiting the reassembly of intercellular junctions of Caco-2 cell monolayers. This study also suggests that other EC domains in addition to EC1 may be involved in cadherin-cadherin interactions in intercellular junctions. In the future, the modulatory activity of cadherin peptides (i.e., HAV6 and ADT6) will be optimized by mutation of the amino acid residues as well as the formation of cyclic peptides. The binding mechanism of cadherin peptides to various domains of E-cadherin will be determined using the expressed EC domains. The pore size of the intercellular junctions of BBB created by cadherin peptides will be determined using the polyethylene glycol (PEG) profiling technique. Finally, the applicability of cadherin peptides for improving in vivo permeation of drugs through the intestinal mucosa will also be evaluated using an in situ rat intestinal mucosa perfusion model.

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References

1. Lutz KL, Siahaan TJ. Molecular structure of the apical junction complex and its contribution to the paracellular barrier. *J Pharm Sci.* 1997; 86(9):977–984. [PubMed: 9294808]

2. Zheng K, Laurence JS, Kuczera K, Verkhivker G, Middaugh CR, Siahaan TJ. Characterization of multiple stable conformers of the EC5 domain of E-cadherin and the interaction of EC5 with E-cadherin peptides. *Chem Biol Drug Des.* 2009; 73(6):584–598. [PubMed: 19635050]
3. Madara JL. Regulation of the movement of solutes across tight junctions. *Annu Rev Physiol.* 1998; 60:143–159. [PubMed: 9558458]
4. Shin K, Fogg VC, Margolis B. Tight junctions and cell polarity. *Annu Rev Cell Dev Biol.* 2006; 22:207–235. [PubMed: 16771626]
5. Mitic LL, Anderson JM. Molecular architecture of tight junctions. *Annu Rev Physiol.* 1998; 60:121–142. [PubMed: 9558457]
6. Gumbiner BM. Regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol.* 2005; 6(8):622–634. [PubMed: 16025097]
7. Takeichi M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science.* 1991; 251(5000):1451–1455. [PubMed: 2006419]
8. Loscher W, Potschka H. Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx.* 2005; 2(1):86–98. [PubMed: 15717060]
9. Dauchy S, Dutheil F, Weaver RJ, Chassoux F, Daumas-Duport C, Couraud PO, Scherrmann JM, De Waziers I, Decleves X. ABC transporters, cytochromes P450 and their main transcription factors: expression at the human blood-brain barrier. *J Neurochem.* 2008; 107(6):1518–1528. [PubMed: 19094056]
10. Gumbiner B, Simons K. A functional assay for proteins involved in establishing an epithelial occluding barrier: identification of a uvomorulin-like polypeptide. *J Cell Biol.* 1986; 102(2):457–468. [PubMed: 3511070]
11. Cerejido M, Shoshani L, Contreras RG. Molecular physiology and pathophysiology of tight junctions. I. Biogenesis of tight junctions and epithelial polarity. *Am J Physiol Gastrointest Liver Physiol.* 2000; 279(3):G477–G482. [PubMed: 10960345]
12. Makagiansar IT, Avery M, Hu Y, Audus KL, Siahaan TJ. Improving the selectivity of HAV-peptides in modulating E-cadherin-E-cadherin interactions in the intercellular junction of MDCK cell monolayers. *Pharm Res.* 2001; 18(4):446–453. [PubMed: 11451030]
13. Sinaga E, Jois SD, Avery M, Makagiansar IT, Tambunan US, Audus KL, Siahaan TJ. Increasing paracellular porosity by E-cadherin peptides: discovery of bulge and groove regions in the EC1-domain of E-cadherin. *Pharm Res.* 2002; 19(8):1170–1179. [PubMed: 12240943]
14. Tamura K, Shan WS, Hendrickson WA, Colman DR, Shapiro L. Structure-function analysis of cell adhesion by neural (N-) cadherin. *Neuron.* 1998; 20(6):1153–1163. [PubMed: 9655503]
15. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell.* 1996; 84(3):345–357. [PubMed: 8608588]
16. Nose A, Tsuji K, Takeichi M. Localization of specificity determining sites in cadherin cell adhesion molecules. *Cell.* 1990; 61(1):147–155. [PubMed: 2317870]
17. Shimoyama Y, Takeda H, Yoshihara S, Kitajima M, Hirohashi S. Biochemical characterization and functional analysis of two type II classic cadherins, cadherin-6 and -14, and comparison with E-cadherin. *J Biol Chem.* 1999; 274(17):11987–11994. [PubMed: 10207020]
18. Niessen CM, Gumbiner BM. Cadherin-mediated cell sorting not determined by binding or adhesion specificity. *J Cell Biol.* 2002; 156(2):389–399. [PubMed: 11790800]
19. Duguay D, Foty RA, Steinberg MS. Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. *Dev Biol.* 2003; 253(2):309–323. [PubMed: 12645933]
20. Takasato Y, Rapoport SI, Smith QR. An in situ brain perfusion technique to study cerebrovascular transport in the rat. *Am J Physiol.* 1984; 247(3 Pt 2):H484–H493. [PubMed: 6476141]
21. Kobayashi N, Ikesue A, Majumdar S, Siahaan TJ. Inhibition of e-cadherin-mediated homotypic adhesion of Caco-2 cells: a novel evaluation assay for peptide activities in modulating cell-cell adhesion. *J Pharmacol Exp Ther.* 2006; 317(1):309–316. [PubMed: 16371447]
22. Liu X, Tu M, Kelly RS, Chen C, Smith BJ. Development of a computational approach to predict blood-brain barrier permeability. *Drug Metab Dispos.* 2004; 32(1):132–139. [PubMed: 14709630]
23. Duizer E, van der Wulp C, Versantvoort CH, Groten JP. Absorption enhancement, structural changes in tight junctions and cytotoxicity caused by palmitoyl carnitine in Caco-2 and IEC-18 cells. *J Pharmacol Exp Ther.* 1998; 287(1):395–402. [PubMed: 9765361]

24. Ma L, Kuang K, Smith RW, Rittenband D, Iserovich P, Diecke FP, Fischbarg J. Modulation of tight junction properties relevant to fluid transport across rabbit corneal endothelium. *Exp Eye Res.* 2007; 84(4):790–798. [PubMed: 17320078]
25. Hochman JH, Fix JA, LeCluyse EL. In vitro and in vivo analysis of the mechanism of absorption enhancement by palmitoylcarnitine. *J Pharmacol Exp Ther.* 1994; 269(2):813–822. [PubMed: 8182550]
26. Knipp GT, Ho NF, Barsuhn CL, Borchardt RT. Paracellular diffusion in Caco-2 cell monolayers: effect of perturbation on the transport of hydrophilic compounds that vary in charge and size. *J Pharm Sci.* 1997; 86(10):1105–1110. [PubMed: 9344165]
27. Artursson P, Magnusson C. Epithelial transport of drugs in cell culture. II: Effect of extracellular calcium concentration on the paracellular transport of drugs of different lipophilicities across monolayers of intestinal epithelial (Caco-2) cells. *J Pharm Sci.* 1990; 79(7):595–600. [PubMed: 2118955]
28. Salama NN, Eddington ND, Fasano A. Tight junction modulation and its relationship to drug delivery. *Adv Drug Deliv Rev.* 2006; 58(1):15–28. [PubMed: 16517003]
29. Chikhale EG, Burton PS, Borchardt RT. The effect of verapamil on the transport of peptides across the blood-brain barrier in rats: kinetic evidence for an apically polarized efflux mechanism. *J Pharmacol Exp Ther.* 1995; 273(1):298–303. [PubMed: 7714780]
30. Pal D, Audus KL, Siahaan TJ. Modulation of cellular adhesion in bovine brain microvessel endothelial cells by a decapeptide. *Brain Res.* 1997; 747(1):103–113. [PubMed: 9042533]
31. Lutz KL, Siahaan TJ. Modulation of the cellular junction protein E-cadherin in bovine brain microvessel endothelial cells by cadherin peptides. *Drug Delivery.* 1997; 4:187–193.
32. Lutz KL, Szabo LA, Thompson DL, Siahaan TJ. Antibody recognition of peptide sequences from the cell-cell adhesion proteins: N- and E-cadherins. *Pept Res.* 1996; 9(5):233–239. [PubMed: 9000249]
33. Blaschuk OW, Sullivan R, David S, Pouliot Y. Identification of a cadherin cell adhesion recognition sequence. *Dev Biol.* 1990; 139(1):227–229. [PubMed: 2328837]
34. Noe V, Willems J, Vandekerckhove J, Roy FV, Bruyneel E, Mareel M. Inhibition of adhesion and induction of epithelial cell invasion by HAV-containing E-cadherin-specific peptides. *J Cell Sci.* 1999; 112(Pt 1):127–135. [PubMed: 9841909]
35. Alattia JR, Kurokawa H, Ikura M. Structural view of cadherin-mediated cell-cell adhesion. *Cell Mol Life Sci.* 1999; 55(3):359–367. [PubMed: 10336300]
36. Gumbiner B, Stevenson B, Grimaldi A. The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J Cell Biol.* 1988; 107(4):1575–1587. [PubMed: 3049625]
37. Gumbleton M, Audus KL. Progress and limitations in the use of in vitro cell cultures to serve as a permeability screen for the blood-brain barrier. *J Pharm Sci.* 2001; 90(11):1681–1698. [PubMed: 11745727]
38. Takeichi M. Cadherins: a molecular family important in selective cell-cell adhesion. *Annu Rev Biochem.* 1990; 59:237–252. [PubMed: 2197976]
39. Zheng K, Middaugh CR, Siahaan TJ. Evaluation of the physical stability of the EC5 domain of E-cadherin: effects of pH, temperature, ionic strength, and disulfide bonds. *J Pharm Sci.* 2009; 98(1):63–73. [PubMed: 18428798]
40. Takeichi M. Functional correlation between cell adhesive properties and some cell surface proteins. *J Cell Biol.* 1977; 75(2 Pt 1):464–474. [PubMed: 264120]
41. Koch AW, Pokutta S, Lustig A, Engel J. Calcium binding and homoassociation of E-cadherin domains. *Biochemistry.* 1997; 36(25):7697–7705. [PubMed: 9201910]
42. Castillo AM, Lagunes R, Urban M, Frixione E, Meza I. Myosin II-actin interaction in MDCK cells: role in cell shape changes in response to Ca²⁺ variations. *J Muscle Res Cell Motil.* 1998; 19(5):557–574. [PubMed: 9682142]
43. Frixione E, Lagunes R, Ruiz L, Urban M, Porter RM. Actin cytoskeleton role in the structural response of epithelial (MDCK) cells to low extracellular Ca²⁺. *J Muscle Res Cell Motil.* 2001; 22(3):229–242. [PubMed: 11763195]

44. Lagunes R, Ruiz L, Frixione E. Contraction of epithelial (MDCK) cells in response to low extracellular calcium is dependent on extracellular sodium. *J Muscle Res Cell Motil.* 1999; 20(8): 761–770. [PubMed: 10730579]
45. Citi S. Protein kinase inhibitors prevent junction dissociation induced by low extracellular calcium in MDCK epithelial cells. *J Cell Biol.* 1992; 117(1):169–178. [PubMed: 1556151]
46. Citi S, Denisenko N. Phosphorylation of the tight junction protein cingulin and the effects of protein kinase inhibitors and activators in MDCK epithelial cells. *J Cell Sci.* 1995; 108(Pt 8): 2917–2926. [PubMed: 7593331]
47. Denisenko N, Burighel P, Citi S. Different effects of protein kinase inhibitors on the localization of junctional proteins at cell-cell contact sites. *J Cell Sci.* 1994; 107(Pt 4):969–981. [PubMed: 8056850]
48. Lewis JE, Jensen PJ, Johnson KR, Wheelock MJ. E-cadherin mediates adherens junction organization through protein kinase C. *J Cell Sci.* 1994; 107(Pt 12):3615–3621. [PubMed: 7706410]
49. Rajasekaran AK, Hojo M, Huima T, Rodriguez-Boulant E. Catenins and zonula occludens-1 form a complex during early stages in the assembly of tight junctions. *J Cell Biol.* 1996; 132(3):451–463. [PubMed: 8636221]
50. Nigam SK, Rodriguez-Boulant E, Silver RB. Changes in intracellular calcium during the development of epithelial polarity and junctions. *Proc Natl Acad Sci U S A.* 1992; 89(13):6162–6166. [PubMed: 1631104]
51. Volberg T, Geiger B, Kartenbeck J, Franke WW. Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca^{2+} ions. *J Cell Biol.* 1986; 102(5):1832–1842. [PubMed: 3084500]
52. Stuart RO, Sun A, Panichas M, Hebert SC, Brenner BM, Nigam SK. Critical role for intracellular calcium in tight junction biogenesis. *J Cell Physiol.* 1994; 159(3):423–433. [PubMed: 8188760]

Figure 1.

Total amount of ^{14}C -mannitol in the brain after *in situ* perfusion. Rat brains were perfused with vehicle or peptide at a flow rate of 10.0 ml/min for 4 min followed immediately by ^{14}C -mannitol (10 μCi) at a flow rate of 10.0 ml/min for 4 min. Experiments were done in triplicate and data are represented as mean $\pm$ S.D. * $p < 0.05$ compared to the vehicle.

Figure 2.

Effect of HAV derivatives on Caco-2 cell monolayers viability. Caco-2 cell monolayers were treated with increasing concentrations of HAV peptides as well as controls for 24 h under 5% CO₂ at 37°C and cell viability was monitored with an MTT assay. No significant cytotoxicity was observed when the cells were exposed to peptide concentrations as high as 4 mM.

Figure 3.

Total amount of ^3H -daunomycin in the brain after *in situ* perfusion. Rat brains were perfused with vehicle or peptide (1 mM) with or without verapamil (500 μM) at a flow rate of 5.0 mL/min for 4 min followed immediately by ^3H -daunomycin (15 μCi) with or without verapamil (500 μM) at a flow rate of 5.0 mL/min for 4 min. (A) Vehicle + ^3H -daunomycin, (B) HAVscr + ^3H -daunomycin, (C) HAV6 + ^3H -daunomycin, (D) Vehicle + ^3H -daunomycin with verapamil, (E) HAV6 + ^3H -daunomycin with verapamil, and (F) HAV6 with verapamil + ^3H -daunomycin with verapamil. Experiments were done in triplicate and data are represented as mean $\pm$ S.D. * p < 0.05 and ** p < 0.01 compared to vehicle.

4A

4B

Figure 4.

Concentration-dependent effects on the inhibition of resealing of intercellular junctions by HAV10, ADT10 (or BLG1), and ADT6 peptides applied to the AP-BL sides concurrently in conditioned Caco-2 monolayers. (A) The HAV10 peptide blocks the resealing the intercellular junctions at 1.0 mM but not at 0.5 mM. (B) ADT10 (BLG1) peptide suppresses the resealing of the intercellular junction at both 1.0 mM and 0.5 mM concentrations. (C) ADT6 blocked the resealing of the intercellular junctions in a concentration-dependent manner (0.5 mM and 1.0 mM).

Figure 5.

Comparison of the activities of BLG1, BLG2, BLG3, and BLG4 peptides (1.0 mM) in inhibition of the resealing of the tight junctions of Caco-2 cell monolayers compared to controls (blank, HAVscr, HAVrev). The peptides were added to both the apical and the basolateral sides. The inhibitory activity was determined by measuring the difference in the magnitude of change in TEER values from 2-h to 8-h time points. In this case, the smaller the change in TEER values the more potent was the peptide in inhibiting the resealing of the tight junctions. Treatment of the cell monolayers with no peptide (blank) and VAA peptide changed TEER values around 25%, and they served as controls. HAVscr and HAVrev did not show significant differences ($p > 0.05$) in changes in TEER values with blank treatment. BLG1, BLG2, and BLG4 significantly inhibited the resealing of the intercellular junctions of Caco-2 cell monolayers compared to blank treatment ($p < 0.05$). In contrast, BLG2 did not inhibit the resealing of the tight junctions.

Figure 6.

Concentration-dependent inhibition of Caco-2 single cell adhesion by ADT10 (BLG1), BLG2, BLG3, BLG4, and anti-E-cadherin antibody (DECMA-1). The results show that ADT10, BLG2, and BLG4 significantly inhibited the single cell adhesion in a concentration-dependent manner compared to untreated control (** $p < 0.01$). In this study, Caco-2 cell monolayers were pretreated in Ca²⁺-free EBBS for 2 h. Then, the cells were incubated with BCECF-labeled Caco-2 single cells for 2 h in the presence of the indicated concentrations of BLG peptides. The data are presented as Ca²⁺-dependent adhesion; this was calculated by subtracting the adhesion under Ca²⁺-deficient condition. The results are expressed as the mean $\pm$ S.D. ($n = 3$). Statistical significance was determined by Dunnett test. There are significant differences vs. control (without peptide); ** $p < 0.01$.

Figure 7. Time-dependent inhibition of re-aggregation of single Caco-2 cells by ADT10 (BLG1). (A) Re-aggregation of Caco-2 single cells after 1 h incubation with 2.0 mM calcium in the absence peptides shows the cells beginning to form monolayers, (B) presence of 1.0 mM of ADT10 (BLG1) for 1 h, or (C) Reaggregation of single cells upon incubation with 1.0 mM BLG1 for 5 h.

Table 1

Sequence of Synthetic Peptides

Peptide Name	Sequence	MW
HAV10	Ac-LFSHAVSSNG-NH ₂	1059
HAV6	Ac-SHAVSS-NH ₂	628
ADT10 (BLG1)	Ac-QGADTPPVGV-NH ₂	981
ADT6	Ac-ADTPPV-NH ₂	640
BLG2	Ac-QDPELPDKNM-NH ₂	1227
BLG3	Ac-NDDGGQFVVT-NH ₂	1092
BLG4	Ac-TYRIWRDTAN-NH ₂	1336
VVA	Ac-VVA-NH ₂	329
HAVscr	Ac-HSVSAS-NH ₂	628
HAVrev	Ac-SSVAHS-NH ₂	628
LABL6	EATDSG	620