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Modulation of Blood–Brain Barrier Permeability in Mice Using Synthetic E-Cadherin Peptide

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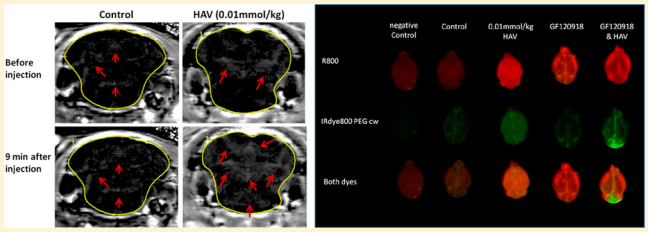
ABSTRACT: The present work characterizes the effects of synthetic E-cadherin peptide (HAV) on blood–brain barrier (BBB) integrity using various techniques including magnetic resonance imaging (MRI) and near-infrared fluorescent imaging (NIRF). The permeability of small molecular weight permeability marker gadolinium diethylenetriaminepentaacetate (Gd-DTPA) contrast agent, the large molecular weight permeability marker, IRDye 800CW PEG, and the P-glycoprotein (P-gp) efflux transporter contrast agent, rhodamine 800 (R800), were examined in the presence and absence of HAV peptide. The results consistently demonstrated that systemic iv administration of HAV peptide resulted in a reversible disruption of BBB integrity and enhanced the accumulation of all the dyes examined. The magnitude of increase ranged from 2-fold to 5-fold depending on the size and the properties of the permeability markers. The time frame for BBB disruption with HAV peptide was rapid, occurring within 3–6 min following injection of the peptide. Furthermore, modulation of BBB permeability was reversible with the barrier integrity being restored within 60 min of the injection. The increased BBB permeability observed following HAV peptide administration was not attributable to changes in cerebral blood flow. These studies support the potential use of cadherin peptides to rapidly and reversibly modulate BBB permeability of a variety of therapeutic agents.



KEYWORDS: *blood–brain barrier (BBB), permeability, magnetic resonance imaging (MRI), E-cadherin peptide (HAV), near infrared fluorescence imaging (NIRF), gadolinium diethylenetriaminepentaacetate (Gd-DTPA) contrast agent, rhodamine 800 (R800), IRDye 800CW PEG, P-glycoprotein (P-gp)*

INTRODUCTION

The approval rate for drugs to treat central nervous system (CNS) disorders is significantly lower than that for other therapeutic classes.[1] Despite aggressive efforts, many new compounds targeting the CNS are not effective in delivering a safe and efficacious dose to the brain.[1,2] One challenging aspect of CNS drug development is the requirement of the compound to cross the blood–brain barrier (BBB) in sufficient amounts for therapeutic response. The BBB consists of a continuous layer of endothelial cells, surrounded by astrocyte foot-processes, and scattered pericytes. An intact BBB presents a formidable obstacle for the entry of drugs into the brain. The brain microvessel endothelial cells that form the BBB have complex tight junctions, low endocytic activity, and an absence of fenestrations[3] that prevent the passage of most polar and hydrophilic solutes from the blood into the brain. Furthermore, numerous efflux transporters including breast cancer resistance protein (BCRP), P-glycoprotein (P-gp), and multidrug resistance protein (MRP) are expressed within the brain microvessel endothelial cells[4] Collectively, these efflux transporters move a wide variety of compounds including phospholipids, ions, peptides, steroids, polysaccharides, amino acids, organic anions, bile acids, drugs, and other xenobiotic from the brain to the blood.[5−10]

Numerous approaches have been examined to enhance drug delivery to the brain. One such method is to chemically modify the drug molecules to reduce their interactions with efflux transporter proteins and, thus, enhance their permeation through the transcellular route.[11] This method had been tested with some chemotherapeutic drugs including paclitaxel. While chemical modification of functional groups has proven beneficial in improving BBB permeability through limiting drug interaction with efflux transporters,[12,13] there are clearly structural limitations to this approach. Another strategy to enhance drug delivery to the brain involves the direct bypassing of the BBB altogether. Two common techniques that utilize this approach include intraventricular infusion of the drug and intracerebral implants containing drug. These techniques have great potential in delivering a high dose of drug locally into the brain without causing systemic side effects; however, when used in clinical settings, the results to date have been disappointing. This may be due to the relatively small surface area of the brain that is in contact with the ventricular compartment and the limited diffusion of the drugs into the brain parenchyma from

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the infusion site in humans compared to preclinical animal models.[14]

The most clinically proven approach to increase drug penetration into the CNS thus far is to reversibly disrupt the junctions formed by the endothelial cells to enhance their permeation through intercellular junctions. This can be accomplished through the use of osmotic agents or pharmacologically through the targeting of membrane receptors that alter BBB permeability. While osmotic agents like mannitol have been used to modulate BBB permeability in both preclinical and clinical setting,[14] the major drawback with osmotic disruption is the long recovery period required for re-establishment of BBB integrity.[15] With several hours required for the return of normal BBB function, patients are susceptible to toxicity.[15] On the other hand, pharmacological agents such as Cereport, a bradykinin receptor agonist, have been shown to transiently disrupt the BBB in various animal models.[14,16] However these agents have failed to produce the desired response in clinical trials due to nonuniform disruption of the BBB.[16] The limitations in clinical studies clearly highlight the need for an alternative method that can provide a homogeneous disruption of the BBB within a desired therapeutic window to maximize the therapeutic response of drugs in the brain.

An important component of the BBB is the adheren junction which is primarily composed of cadherin proteins. The binding of cadherin proteins on adjacent brain microvessel endothelial cells forms a homolytic dimer within the cell junction that limits the paracellular passage of solutes with diameter greater than 11 Å or approximately 500 Da.[11,17] The cadherin protein has an extracellular (EC) domain which consists of five tandem repeated units (EC-1 to EC-5). Site directed mutagenesis studies have shown that the highly conserved region of His-Ala-Val (HAV) is involved in the formation of the dimer. Synthetic peptides based on the HAV region sequence have also been shown to inhibit the interactions between the E-cadherin molecules and prevent the aggregation of bovine brain microvessel endothelial cells in a concentration dependent manner.[18] Furthermore, pretreatment of Madin Darby canine kidney (MDCK) with a HAV based peptide having the amino acid sequence of Ac-SHAVSS-NH_2 resulted in an increased paracellular diffusion of radiolabeled mannitol and decreased transepithelial electrical resistance (TEER).[19] Based on these *in vitro* studies, the flanking of HAV residue can directly interfere with the interactions between the cadherin proteins. Furthermore, HAV peptide has been shown to enhance the brain delivery of ^{14}C-mannitol and ^{3}H-daunomycin in an *in situ* rat brain perfusion model.[20] However, the extent of BBB disruption produced by HAV peptides has not been fully elucidated *in vivo*. The present studies set out to examine the effects of HAV peptide on BBB permeability *in vivo* with special emphasis on the time to onset, and duration of action for HAV peptide-induced changes in cerebral vascular permeability in the mouse. Furthermore, using both magnetic resonance imaging (MRI) and near-infrared fluorescence (NIRF) contrast agents, alterations in BBB permeability to small and large molecular weight compounds as well as a P-glycoprotein substrate were evaluated. The results demonstrate that HAV peptide increased BBB permeability. Modulation of BBB permeability was rapid, occurring within minutes following systemic administration of HAV peptide. Equally important, BBB integrity was restored within 1 h. The modulation of BBB permeability with HAV peptide was observed for both small and large molecular weight permeability agents and, to a lesser extent, with P-gp dependent permeability agents. The alterations in BBB permeability occurred without any disruption in cerebral blood flow. Together these studies suggest cadherin peptides can be used to transiently modulate BBB permeability for enhanced drug delivery to the brain.

■ MATERIALS AND METHODS

The HAV peptide (Ac-SHAVSS-NH_2) was synthesized using solid phase method with Fmoc-chemistry in Pioneer peptide synthesizer. After removal from the resin, the peptide was purified using a semipreparative C18 column in HPLC. The pure fractions were pooled and lyophilized. The purity of the peptide is higher than 96% as determined by C18 analytical HPLC. The identity of the peptide was confirmed by mass spectrometry. The NIRF imaging agents, IRDye 800CW PEG and rhodamine 800 (R800), were obtained from Licor (Lincoln, NE) and Exciton (Dayton, OH), respectively. Gadolinium diethylenetriaminepentaacetate (Gd-DTPA) contrast agent used for MRI of BBB permeability was obtained from Berlex (Lachine, QC, Canada). Ketamine hydrochloride and xylazine were purchased from Wyeth (Guelph, ON, Canada) and Bayer Inc. (Toronto, ON, Canada), respectively. All other reagents and chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

HAV Response *in Vivo*. Adult female Balb/c mice were used to characterize the BBB-disruption profile produced by HAV peptide. Mice were obtained from the University of Manitoba breeding colony and maintained in the Central Animal Care Facility under temperature-controlled environment with 12 h dark/light cycle and unlimited access to food and water. All animal experiments followed the Canadian Council on Animal Care (CCAC) guidelines and were approved by the University of Manitoba Animal Care Committee (protocol number 09-049). Quantitative determination of BBB permeability was performed using three different imaging agents and magnetic resonance (MRI) and near-infrared fluorescence (NIRF) imaging modalities described below.

Magnetic Resonance Imaging of BBB Permeability. The onset and duration of HAV peptide-induced alterations in BBB permeability were initially assessed using magnetic resonance imaging (MRI) with Gd-DTPA contrast agent as described previously by On et al.[21] Mice were anesthetized and secured in a Bruker Biospect MR (7 T/21 cm spectrometer with 2.5 × 2.5 cm^2 field of view). A series of T1-weighted images (rare factor = 8, echo time = 11.56 ms, effective echo time = 11.56 ms, repetition time = 852 ms, averages = 6, total image time = 2.40 min) and T2-weighted images (rare factor = 8, echo time = 20 ms, effective echo time = 80 ms, repetition time = 1640 ms, averages = 12, total image time = 10.50 min) of the mouse brain were obtained prior to administration of Gd-DTPA contrast agent to acquire background images of the mouse brain. The onset of HAV induced BBB disruption in mice was determined by administration of Gd-DTPA contrast agent (0.4 mmol/kg) together with HAV (0.001−0.032 mmol/kg) or vehicle (PBS) via tail vein injection. A series of T1-weighted images were obtained immediately following Gd-DTPA administration and at 3 min intervals throughout a 21 min imaging session. After 21 min, a second dose of Gd-DTPA was administered and T1-weighted images were obtained at 3 min intervals for an additional 21 minute imaging session. To confirm the BBB disruption period for HAV peptide, a separate

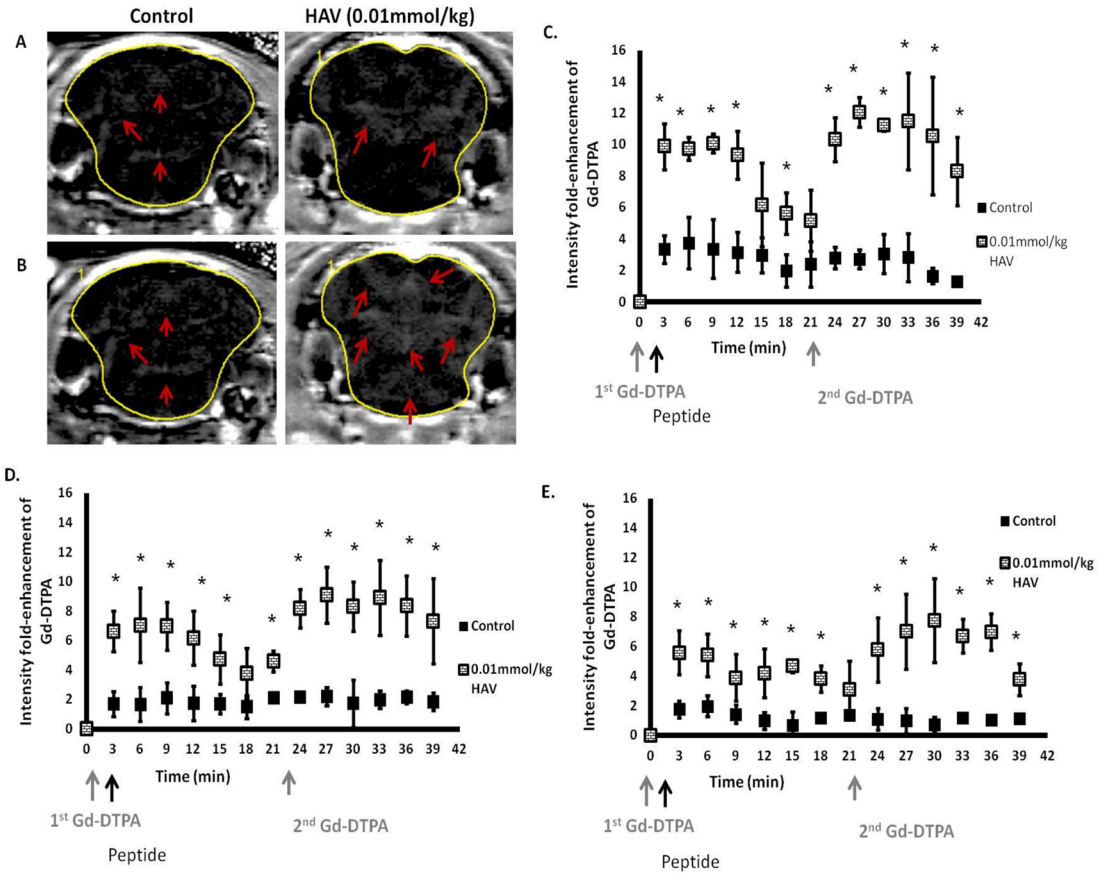

Figure 1. Assessment of HAV peptide-mediated effects on BBB permeability using Gd-DTPA contrast-enhanced MRI. Representative T1-weighted posterior coronal slice images of mouse at time 0 (A) and 9 min (B) after an iv injection of either vehicle (PBS) or (0.01 mmol/kg) HAV peptide. Quantitative analysis of pixel intensity of Gd-DTPA in (C) posterior brain region, (D) midbrain region, and (E) anterior brain region following an administration of either vehicle (PBS) or (0.01 mmol/kg) HAV peptide solution. Red arrows indicate regions of enhancement. Data is expressed as the fold-enhancement of whole brain pixel intensity (outlined in yellow) at a particular time point compared to the whole brain (outlined in yellow) pixel intensity at the time 0. * $p < 0.05$ compared to control mice at the same time point. Values represent the mean ± SEM for 4 mice per treatment group.

group of mice were administered HAV peptide (0.01 mmol/kg) or vehicle 1 h prior to the first Gd-DTPA (0.4 mmol/kg) injection. Immediately following administration of Gd-DTPA contrast agent, a series of T1-weighted scans were taken at an interval of 3 min for a period of 21 min. After the first 21 min of scanning, the mice received a second dose of Gd-DTPA and were imaged for an additional 21 min as described above. Quantitative assessment of Gd-DTPA enhancement in the brain was accomplished by manually outlining regions of interest (ROI) within the coronal brain slices using Marevisi 7.2 software (Institute for Biodiagnostics, National Research Council, Canada). Changes in Gd-DTPA intensity in the brain were determined using a percent difference analysis of brain slice images within the Paravision 3.0 software package according to the following formulas:[22]

$$((\text{post-Gd-DTPA T1-weighted images} - \text{pre-Gd-DTPA T1-weighted images}) \div \text{pre-Gd-DTPA T1-weighted images}) \times 100$$

The resulting data were graphed as the fold-enhancement in Gd-DTPA at the various time intervals.

Magnetic Resonance Imaging of Cerebral Blood Flow (CBF). In a separate study, the effects of HAV on cerebral blood flow were also examined using perfusion-weighted MRI with a Bruker Biospec 7 T/21 cm spectrometer (Bruker BioSpin, Karlsruhe, Germany) equipped with a 2.5 cm diameter quadrature volume coil (National Research Council, Winnipeg, MB, Canada). Mice were anesthetized with 1.5 to 2% isoflurane in 30% oxygen and 70% nitrous oxide and were secured in the magnet and maintained at a core temperature constant at 37 °C. The relative CBF was measured using an adiabatic spin labeling sequence with a 36-echo HASTE readout following a 400 ms post tagging delay at 1 mm slice thickness with a resolution of 234 μm. Coronal slices images of the brain were obtained at 3.4 mm below the bregma for CBF measurement. Analysis of MRI CBF was performed using Marivisi 7.2 analysis software (National Research Council, Winnipeg, MB, Canada). The relative blood flow was measured throughout the brain region and was expressed as the percent difference in intensity.

Near Infrared Fluorescence Imaging of BBB Permeability. The effects of HAV peptide on the BBB permeability of a large molecular weight compound as well as a P-glycoprotein (P-gp) sensitive agent was also examined as previously described using NIRF imaging agents.[21,23] For these studies, mice received both IRDye 800CW PEG (0.01 μmol/kg), a pegylated dye of approximately 25 kDa molecular weight used for assessing macromolecule vascular leakage,[24] and R800 (0.032 μmol/kg), a NIRF dye with P-gp substrate properties.[23,24] The NIRF

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probes were administered to mice under four different treatment regimes. In treatment regime A, the mice received only vehicle injection; treatment regime B received only 0.01 mmol/kg HAV; treatment regime C received (9 mg/kg) GF120918, an inhibitor of P-gp; while treatment regime D received a combination of both 0.01 mmol/kg HAV and 9 mg/kg GF120918. The dosage 9 mg/kg of GF120918 was selected based on previous *in vivo* studies showing significant inhibition of P-gp activities without adverse effects on the animals. The mice were sacrificed at various times (15−60 min) following treatment via cardiac perfusion with 10% formaldehyde solution. The brain and other tissues were removed, and the accumulation of NIRF dyes was examined *ex vivo* using an Odyssey near-infrared imaging system (Licor, Lincoln, NE). Quantitative assessment of fluorescence was performed on ROI in 2 mm thick coronal tissue slices and normalized to fluorescence from blood samples taken at the time of tissue collection. Resulting values were presented as relative fluorescence units per mm^2 of tissue divided by relative fluorescence units per microliter of blood.

Statistical Analysis. Student *t* tests were used to analyze the changes in permeability of Gd-DTPA in different brain regions as well as the changes in permeability of IRDye 800CW PEG in various tissues following the systemic administration of either vehicle or HAV (Figure 1 and Figure 6 respectively). The dose dependent effects of HAV on Gd-DTPA accumulations as well as the *ex vivo* accumulations of R800 and IRDye 800CW PEG in the brain were analyzed using ANOVA with Student−Newman−Keul post hoc comparison of the means. Statistical significance was set at $p < 0.05$ unless otherwise stated.

RESULTS

Characterizing of HAV Peptide-Induced Alterations in BBB Permeability Using MRI. Modulation of BBB permeability with HAV peptide was initially assessed using Gd-DTPA contrast enhanced MRI. Figure 1 shows a representative series of T1-weighted MR images taken from the posterior brain region of vehicle (PBS) or 0.01 mmol/kg HAV peptide-treated mice. Mice receiving vehicle had no change in BBB permeability as shown by the similar Gd-DTPA contrast enhanced images of coronal brain slice at time 0 (prior to the injection of vehicle solution) and at 9 min (time 9) following vehicle injection (Figure 1A and Figure 1B, respectively). In contrast, administration of 0.01 mmol/kg of HAV peptide resulted in an increased accumulation of the Gd-DTPA contrast agent in the brain (represented by white-gray appearance indicated by red arrows) when compared to images obtained in the same mouse prior to HAV peptide injection at time 0 (Figure 1). This increase in BBB integrity in response to HAV peptide was observed in all regions of the brain examined (Figure 1C−E). Quantitative assessment of Gd-DTPA contrast enhancement in the various brain regions indicated an approximately 2−4-fold increase in Gd-DTPA intensity in the HAV treatment group compared to control mice in all regions of the brain examined (Figure 2A). Furthermore, the disruption of BBB integrity mediated by HAV peptide was dose dependent with the lowest dose (0.001 mmol/kg) having no effects on Gd-DTPA accumulation in the brain and the highest dose (0.032 mmol/kg) producing a 4-fold higher enhancement of the Gd-DTPA signal in the brain compared to control mice (Figure 2B).

The time frame for BBB disruption with the HAV peptide was also determined using MRI techniques. The increase in

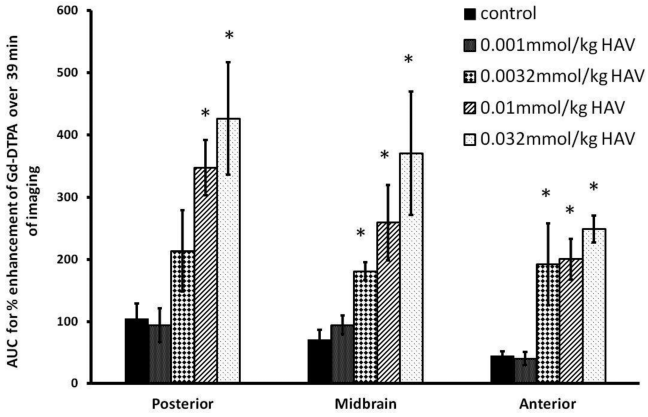

Figure 2. Area under the curve for % Gd-DTPA enhancement over the combined 39 min imaging session in control and various dosages (0.001−0.032 mmol/kg) of HAV peptide treated mice within different brain regions. * $p < 0.05$ compared to control mice in the same region. Values represent the mean ± SEM for 4 mice per treatment group.

Gd-DTPA accumulation in the brain was rapid, with significant increases observed within 3−6 min following systemic administration of HAV peptide (Figure 1). Furthermore, HAV peptide-induced disruption in BBB permeability was transient. This transient nature of the BBB disruption was confirmed as administration of Gd-DTPA contrast agent at 1 h following administration of HAV peptide (0.01 mmol/kg) resulted in no significant increases in Gd-DTPA accumulation in the brain (Figure 3). To confirm that the BBB permeability

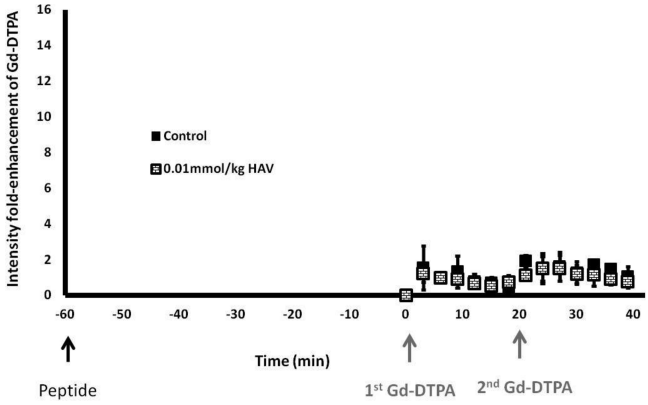

Figure 3. Quantitative analysis of pixel intensity for Gd-DTPA enhancement normalized to the pixel intensity at time 0 following a 60 min pretreatment with either vehicle (PBS) or 0.01 mmol/kg HAV peptide. The analysis was done on the posterior region of the brain. Values represent the mean ± SEM for 4 mice per treatment group.

enhancing affects mediated by HAV were independent of cerebral blood flow, a separate group of mice were selected for cerebral blood flow assessment using MRI. As shown in Figure 4, HAV treatment did not alter the blood flow to the brain as indicated by similar image intensity compared to both preinjection in the same mouse and control mice receiving vehicle alone.

Spectrum of BBB Enhancement Produced with HAV Peptide Treatment. The range of solutes that could be delivered to the brain by HAV peptide modulation of BBB permeability was examined using a large macromolecule paracellular marker (IRDye 800CW PEG) as well as small

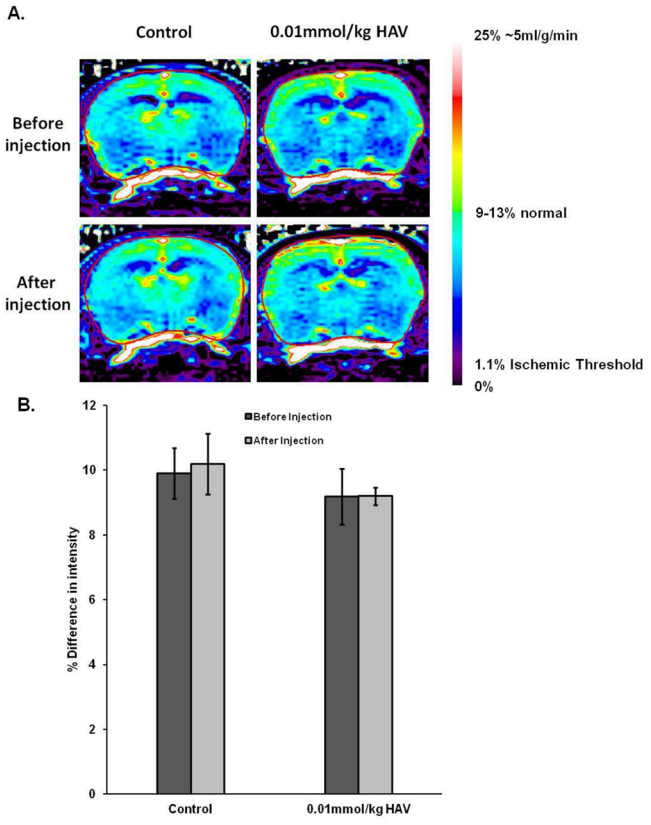

Figure 4. Assessment of cerebral blood flow mediated by 0.01 mmol/kg HAV using MRI. Representive of perfusion weighted MR images obtained before and after an injection of either vehicle (PBS) or 0.01 mmol/kg of HAV via tail vein (A) as well as the quantitative data for CBF obtained from the images (B). Two-way ANOVA was used with treatment and imaging time as the independent variables. There was no statistically significant difference between treatment groups or imaging time, and there was no interaction between the two. Values represent the mean ± SEM for 4 mice per treatment group.

molecule, P-gp substrate (R800). Both probes were examined at different time points (15–60 min) following HAV peptide exposure using near-infrared fluorescent imaging techniques. Under control conditions, there was little accumulation of either IRDye 800CW PEG or R800 in the brain (Figure 5). Indeed most of the fluorescence appeared to be associated with the cerebral vasculature (Figure 5A and Figure 5B) with nonvascular brain regions showing minimal fluorescence (Figure 5A). Similarly, R800 fluorescence activity in the brain under control conditions was comparable to that observed in mice receiving no R800 injections (Figure 5A and Figure 5B). In contrast, mice receiving HAV peptide showed a significant increase in fluorescence of both IRDye 800CW PEG and R800 in the brain tissue (Figure 5). In the case of R800 there was an approximately 2-fold increase in R800 accumulation in the brain following HAV peptide exposure (Figure 5C). This was comparable to the increases in brain accumulation of R800 observed following treatment with the P-gp inhibitor, GF120918 (Figuer 5C). For IRDye 800CW PEG, an approximately 6-fold increase in brain accumulation was observed following HAV peptide treatment (Figure 5D). In contrast, GF120918 had no effect on IRDye 800CW PEG accumulation in the brain (Figure 5D). The effect of the HAV peptide on BBB permeability was transient with maximal increases in both R800 and IRDye 800CW PEG observed within 15 min of HAV peptide exposure and no significant increases over that of control observed following 60 min pretreatment with the HAV peptide (Figure 5B). Examination of fluorescence accumulation in other tissues indicated that systemic exposure to HAV peptide altered permeability of IRDye 800CW PEG in other tissues besides the brain. Those tissues displaying increased permeability of IRDye 800CW PEG permeability marker following HAV treatment included the kidneys, intestines, and lungs (Figure 6).

■ DISCUSSION

One of the most significant obstacles in the development of agents to treat CNS diseases is achieving therapeutically relevant concentrations of drug in the brain due to the presence of the blood–brain barrier (BBB) and the blood cerebral spinal fluid barrier (BCSFB). These barriers are composed of epithelial (BCSFB) or endothelial (BBB) cells with tight junctions and active efflux transporters that together restrict both the paracellular and transcellular passage of solutes into the brain.[25] However, there are several ways to circumvent these barriers including the use of high concentration of osmotic agents or bradykinin analogues to reversibly disrupt the tight junction proteins allowing more drugs to penetrate. While current transient disruption techniques are promising, each has limitations. One major drawback with the use of high concentration of mannitol to transiently disrupt BBB integrity is the long recovery period associated with the disruption, which can last up to several hours, resulting in neurotoxicity and inflammation. Although disruption of BBB integrity with bradykinin analogues occurs over a more condensed time frame, the lack of effectiveness in clinical trials has been attributed in part to the nonuniform distribution of their receptors in the brain, which ultimately resulted in nonuniform distribution of the drugs.[26,27] Clearly the failure of these compounds highlights the need for a new agent with a better BBB disruption profile that is able to uniformly enhance BBB permeability to improve drug delivery to the brain.

Synthetic HAV peptide had been shown to inhibit the homolytic interaction between E-cadherin protein, an essential protein that forms the adherens junctions of the BBB. Indeed the binding of these peptides to the extracellular domain of the proteins has been shown to reduce the TEER reading and enhance the permeability of mannitol across the MDCK cell monolayer.[19] The ability of the HAV peptide to alter BBB has also been demonstrated using a rat *in situ* brain perfusion model.[20] However, the ability of the HAV peptide to alter BBB permeability in the *in vivo* setting has not been demonstrated. The present studies describe a series of experiments detailing the effects of HAV on BBB permeability *in vivo*, specifically focusing on the time to onset, and duration of action for HAV-induced changes in cerebral vascular permeability in the mouse. In the present study, administration of HAV peptide resulted in significant dose-dependent increases in the accumulation of Gd-DTPA in the brain. The HAV peptide effects on BBB permeability were not attributable to alterations in cerebral blood flow as the perfusion-weighted images from MRI were similar in both HAV peptide treated and control mice. The increased BBB permeability to Gd-DTPA observed following HAV administration was consistent with previous *in situ* perfusion study using radiolabeled mannitol, another small molecular weight paracellular marker.[20] The studies by Kiptoo and colleagues demonstrated a significant increase in the accumulation of intravenously infused ^{14}C-mannitol following

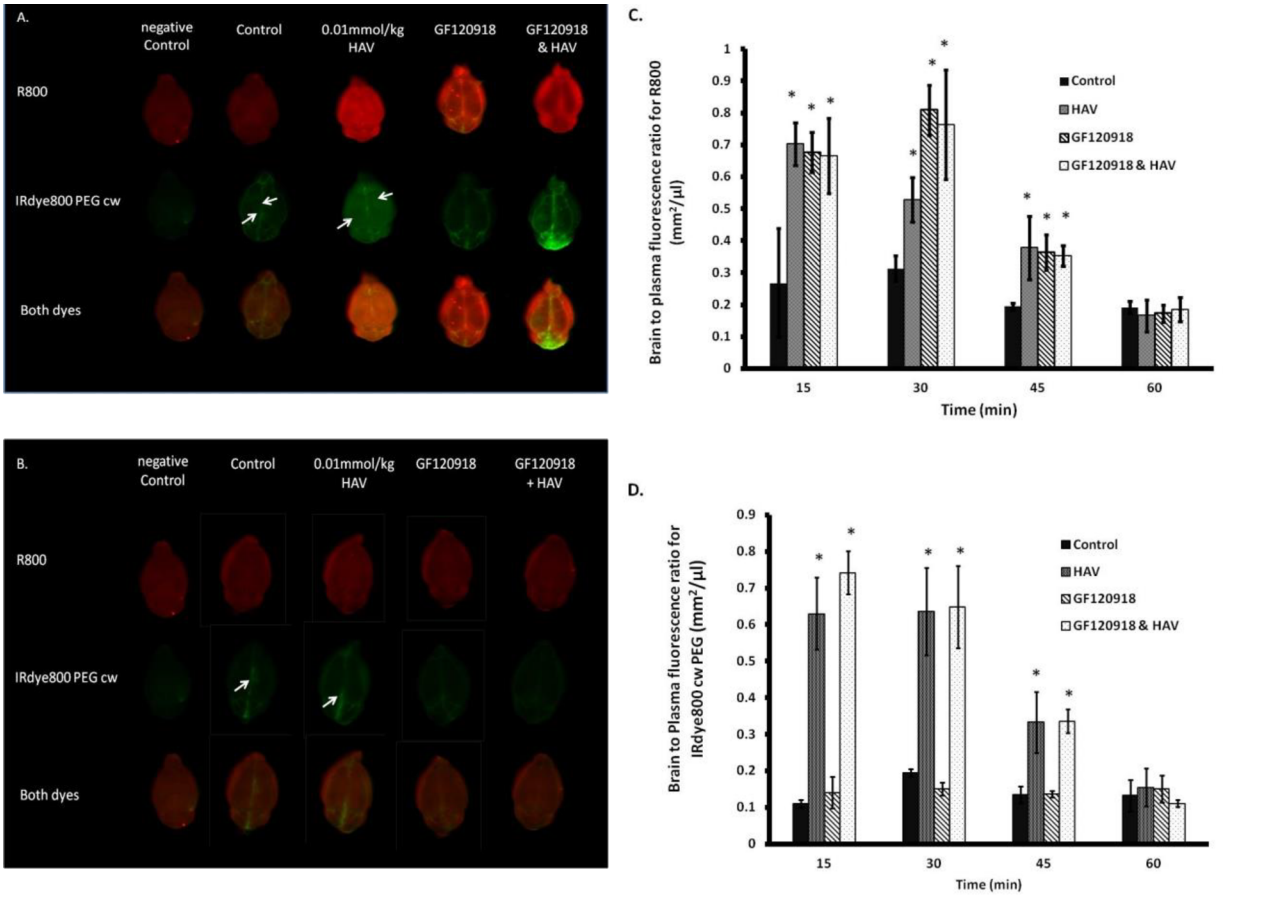

Figure 5. Effects of HAV peptide and GF120918 on the permeability of a P-gp substrate, R800, as well as the permeability of a large macromolecule, IRdye800 cw PEG in the BBB at 15 min (A) and 60 min (B) pretreatment of either vehicle (PBS) and 0.01 mmol/kg HAV peptide. Quantitative assessment of R800 (C) and IRDye 800CW PEG (D) in the brain at various time points under the different treatment groups including vehicle (PBS), GF120918, HAV peptide, and HAV peptide in combination with GF120918. * $p < 0.05$ compared to control mice. Values represent the mean ± SEM for 4 mice per treatment group.

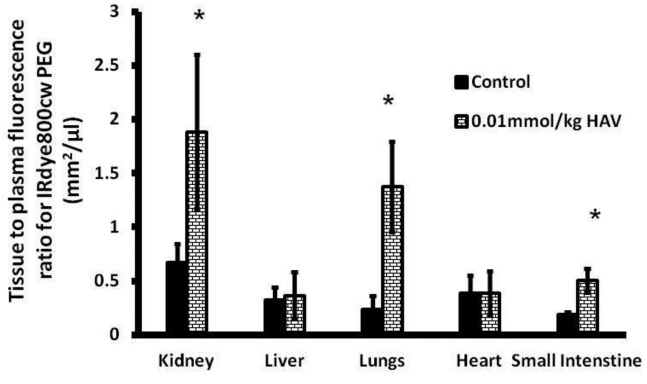

Figure 6. Quantitative assessment of IRDye 800CW PEG in various tissues at 20 min following an injection of the dye normalized to the intensity of IRDye 800CW PEG in the blood . * $p < 0.05$ compared to control mice. Values represent the mean ± SEM for 4 mice per treatment group.

treatment with 1 mM HAV peptide compared to either a vehicle solution or amino acid sequence scrambled control peptide.[20] Furthermore, when the dose of HAV peptide was decreased to 0.5 mM, the amount of ^{14}C-mannitol that accumulated in the brain was reduced by 40%.[20] This is consistence with the dose-dependent effects of HAV peptide observed with Gd-DTPA in the present study.

The Gd-DTPA contrast agent has been widely used in clinical settings to identify localized cerebral microvascular leakage resulting from brain tumors or stroke.[22] However, the present study employed Gd-DTPA contrast enhanced MRI to quantitatively characterize both the time course and magnitude of BBB disruption following HAV peptide exposure throughout the entire brain. This approach has been used recently to characterize transient BBB alterations produced by lysophosphatidic acid (On et al.[21]). An advantage in using MR imaging technology is the fast acquisition time, which allowed the monitoring of rapid changes in BBB permeability in response to HAV-peptide exposure within the same animal. Based on these studies, HAV-induced changes in BBB permeability were apparent within 3 min. Another advantage to this approach is the ability to monitor BBB alterations in various brain regions (i.e., posterior, midbrain, and anterior regions). Consistent with previous studies,[23] regional differences in BBB permeability were apparent with anterior regions of the brain having reduced Gd-DTPA contrast enhancement compared to posterior regions. However, despite the regional differences in baseline BBB permeability, HAV peptide produced similar magnitudes of BBB disruption. The Gd-DTPA contrast enhanced MRI also provided a means for determining the time frame of BBB disruption mediated by HAV peptide. Indeed, this is the first study that examines the disruption time frame of HAV *in vivo*. The observation that Gd-DTPA enhancement is completely

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abolished when the mice were given HAV peptide at 1 h prior to the injection of the contrast agent indicates that BBB integrity was completely restored within one hour of HAV peptide exposure.

The one hour time frame for BBB disruption with the HAV peptide is considerably less than *in vitro* studies reporting the return of barrier properties after 6 h incubation with HAV peptide.[19] Furthermore, *in vitro* HAV peptide stability studies reported a rat plasma half-life of 4.7 h. While various peptidases present in the blood and tissue can influence peptide activity, hepatic uptake and biliary excretion is the major route of elimination of a variety of small linear and cyclic peptide therapeutics.[28,29] Consequently, the shorter period of disruption observed in the present study likely reflects the importance of hepatic clearance mechanisms in determining the circulation time of the HAV peptide. It should be noted that, from a clinical application perspective, the relatively short duration of disruption observed *in vivo* is a desired characteristic for CNS drug delivery applications, as prolonged periods of BBB disruption in the clinical setting can lead to increased incidence of brain inflammation and edema.

The impact of HAV peptide on the pore-size opening of the tight junction of the BBB was also determined by utilizing the near-infrared imaging technology and the dyes rhodamine 800 and IRDye 800CW PEG. Given its lipophilic nature, R800 would normally penetrate the BBB via transcellular diffusion; however, as the dye is also a substrate for P-glycoprotein, one of the main efflux transporter in the BBB, the brain accumulation of R800 is limited.[23] It was previously shown that when P-gp was inhibited by GF120918, the accumulation of R800 in the brain and the choroid plexus was significantly enhanced by 4- and 2-fold respectively.[23] In the present study, treatment with GF120918 significantly increased the accumulation of R800 dye in the brain. The effect of GF120918 on BBB permeability was selective for R800 as the brain penetration of the large molecular weight paracellular marker, IRDye 800CW PEG, was unaffected. It is important to note that treatment with the HAV peptide resulted in similar increases in the accumulation of R800 in the brain as were observed with the P-gp inhibitor, GF120918. The one-hour window for HAV peptide-mediated enhancement of the brain accumulation of R800 was similar to that observed with Gd-DTPA. The ability to increase the brain penetration of R800 suggest that HAV peptide based modulation of BBB permeability could be effective for therapeutics that have reduced BBB permeability due to active cellular efflux transport mechanisms. These findings support the previous studies in the *in situ* brain perfusion model reporting an effect of HAV peptide on the BBB permeability of daunomycin, a P-gp transport substrate.[20]

It is postulated that HAV peptide interferes with the intercellular junctions of the cells allowing for increased R800 permeability in the BBB via the paracellular route rather than the transcellular route. In the present study, the combination of GF120918 and HAV peptide did not produce an additive affect in the amount of R800 accumulation in the brain. As the HAV peptide and P-gp inhibitor are influencing different permeability pathways, one would anticipate potential additive or synergistic effects with the combined treatment. An additive effect of HAV peptide and P-gp inhibition on radiolabeled daunomycin permeability was observed in the *in situ* brain perfusion studies.[20] The results in the present study may be due to a maximal enhancement of R800 in the brain through either increased paracellular diffusion (HAV peptide treatment) or increased transcellular diffusion (P-gp inhibition). If the HAV peptide and GF120918 treatments resulted in maximal increases in R800 accumulation in the brain, then combining the two treatments would have no additional effects.

In addition to increasing the permeability of R800, the disruption of the BBB via HAV also enhanced the diffusion of a large paracellular compound IRDye 800CW PEG. The IRDye 800CW PEG is a large molecular weight near-infrared fluorescence imaging agent that has traditionally been used to examine vascular leakage and lymphatic drainage.[24] With a molecular weight of 25 kDa, little amount of dye was expected to penetrate the brain following a systemic injection under normal conditions. However, when the dye was administered in the presence of HAV, a substantial enhancement of IRDye 800CW PEG leakage was observed in the brain. In addition to the brain, the permeability of IRDye 800CW PEG in other organs including the kidney, the lungs, and the small intestine was also increased in the presence of HAV. In clinical applications, potential off-target site enhancement of vascular permeability would be minimized by carotid artery injections of the HAV peptide that primarily target the cerebral vasculature. As expected, the presence of GF120918 had no impact on the diffusion of this paracellular marker. The results from this study suggested that HAV-induced BBB disruption was not limited to small molecular weight compounds but was also present for larger macromolecules. As the magnitude of increase in small molecule Gd-DTPA penetration was greater than that observed with the large molecule IRDye probe, these findings further support the targeted alteration of paracellular diffusion with HAV peptides.

In summary, using both MRI and NIRF, the present studies showed that HAV peptide was able to increase BBB permeability. Consistent with the binding of cadherin and disruption of tight junction complexes produced by the HAV peptide, increases in BBB permeability were most apparent for the small hydrophilic permeability marker, Gd-DTPA. However, significant increases in BBB permeability were observed following HAV exposure for both the large hydrophilic permeability marker, IRDye 800CW PEG, and the P-glycoprotein dependent permeability marker, R800. The HAV peptide-induced enhancement of BBB permeability was transient with complete restoration of BBB integrity observed within a 60 min time period. The rapid onset and transient nature of the BBB modulation produced with the HAV peptide is well-suited for CNS drug delivery applications.

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Notes

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