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Brain Delivery of Drug and MRI Contrast Agent: Detection and Quantitative Determination of Brain Deposition of CPT-Glu Using LC-MS/MS and Gd-DTPA Using Magnetic Resonance Imaging

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Abstract

Successful treatment and diagnosis of neurological diseases depend on reliable delivery of molecules across the blood-brain barrier (BBB), which restricts penetration of pharmaceutical drugs and diagnostic agents into the brain. Thus, developing new non-invasive strategies to improve drug delivery across the BBB is critically needed. This study was aimed at evaluating the activity of HAV6 peptide (Ac-SHAVSS-NH₂) in improving brain delivery of camptothecin-glutamate (CPT-Glu) conjugate and gadolinium-diethylenetriaminepentaacetate (Gd-DTPA) contrast agent in Sprague-Dawley rats. Brain delivery of both CPT-Glu and Gd-DTPA was evaluated in an *in situ* rat brain perfusion model in the presence and absence of HAV6 peptide (1.0 mM). Gd-DTPA (0.6 mmol/kg) was intravenously (i.v.) administered with and without HAV6 peptide (0.019 mmol/kg) in rats. The detection and quantification of CPT-Glu and Gd-DTPA in the brain were carried out by LC-MS/MS and quantitative magnetic resonance imaging (MRI), respectively. Rats perfused with CPT-Glu in combination with HAV6 had significantly higher deposition of drug in the brain compared to CPT-Glu alone. MRI results also showed that administration of Gd-DTPA in the presence of HAV6 peptide led to significant accumulation of Gd-DTPA in various regions of the brain in both the *in situ* rat brain perfusion and *in vivo* studies. All observations taken together indicate that HAV6 peptide can disrupt the BBB and enhance delivery of small molecules into the brain.

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INTRODUCTION

Many potential drugs developed for the treatment of diseases of the central nervous system (CNS) such as Alzheimer's, Parkinson's, brain tumors and brain infections have failed due to challenges in delivering sufficient therapeutic doses into the brain. Difficulty in delivering drugs is largely attributed to the presence of the blood-brain barrier (BBB) and/or unfavorable physical-chemical properties of the drug for penetrating the BBB.¹⁻⁴ The BBB is comprised of capillary endothelial cells that act as a highly selective filter between systemic blood circulation and the brain. The main function of the BBB is to regulate the passage of nutrients into the brain and provide protection against unwanted invaders such as toxins and pathogens.² The transcellular transport of molecules (e.g., drugs or diagnostic agents) is normally limited by their unfavorable physicochemical and structural properties as substrates for efflux pumps.^{5, 6} Another route to the brain is the paracellular passage, in which drugs can permeate through the intercellular tight junctions of the BBB.^{1, 7} Tight junctions (TJs), adherens junctions (AJs), and desmosomes are composed of cell adhesion proteins that act as zippers between cell membranes. At the TJs, the cell membranes are connected by protein-protein binding of occludins and claudins while the AJs are linked by E- and VE-cadherins that are joined into the cell cytoplasm by the scaffolding proteins α -, β -, and γ -catenins.^{2, 8} Desmocollins and desmogleins are cadherin family proteins located at desmosomes below the AJs and disruption of TJs and AJs has been shown to enhance paracellular permeation of molecules.^{1, 9}

HAV (His-Ala-Val) and ADT (Ala-Asp-Thr) peptides have been developed to modulate cadherin-cadherin interactions at the intercellular junctions and to increase the transport of marker molecules through the paracellular pathway of the BBB.¹⁰⁻¹² HAV and ADT peptides derived from the EC1 domain of E-cadherin caused increases in paracellular permeation of ¹⁴C-mannitol through Madin-Darby canine kidney (MDCK) cell monolayers, and these peptides caused a drop in transepithelial electrical resistance (TEER) values of the cell monolayers.^{13, 14} Furthermore, HAV6 peptide (Ac-SHAVSS-NH₂) enhanced the paracellular permeation of the anticancer drug ³H(G)-daunomycin and ¹⁴C-mannitol across the BBB in an *in situ* brain perfusion rat model.¹⁰ Recently, HAV6 peptide has been shown to enhance *in vivo* delivery of gadolinium-diethylenetriaminepentaacetate (Gd-DTPA), rhodamine 800 (R800), and IRDye 800cw-polyethylene glycol (800cw-PEG, 25 kDa).¹² Gd-DTPA is a magnetic resonance imaging (MRI) contrast agent while R800 is a near IR dye for fluorescence imaging as well as a substrate for the efflux pump P-glycoprotein (Pgp).

The focus of this work was to evaluate the ability of HAV6 peptide to enhance the brain delivery of (a) camptothecin-glutamate (CPT-Glu; Figure 1) in an *in situ* rat brain perfusion model and (b) gadolinium diethylenetriaminepentaacetate (Gd-DTPA) in an *in situ* rat brain

perfusion model and *in vivo* in Sprague-Dawley rats. This work was also aimed at developing methods to detect and quantify the amount of molecules delivered to the brain using liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS) and magnetic resonance imaging (MRI). CPT-Glu is an ester conjugate between an anticancer drug camptothecin (CPT) and L-glutamic acid (L-Glu) to improve solubility of CPT. In the *in situ* rat brain perfusion model, CPT-Glu conjugate was delivered in the presence and absence of HAV6 peptide. To detect the amount of CPT-Glu and CPT in the brain, a method to extract CPT-Glu and CPT from the brain was developed. Then, an LC-MS/MS method was developed to quantitate the amount of CPT-Glu and CPT in the brain extract. The idea of using LC-MS/MS is to directly detect the compound of interest and its metabolites while eliminating the use of radiolabeled compounds. The effect of HAV6 peptide in enhancing the brain delivery of Gd-DTPA was evaluated using *in situ* rat brain perfusion and *in vivo* intravenous (i.v.) delivery in Sprague-Dawley rats and the brain localization and deposition of Gd-DTPA was quantified using MRI.

MATERIALS AND METHODS

Peptide Synthesis and Reagents

The HAV peptide used in this study was synthesized using solid phase peptide synthesis with Fmoc-chemistry as previously reported.^{10, 14} The peptide was cleaved from the resin using a standard method, and purified by reversed-phase HPLC using a C₁₈ semi-preparative column. The pure fractions were pooled and lyophilized. The purity of the peptide used was >96% as determined by analytical HPLC using a C₁₈ analytical column. The identity of the peptide was confirmed by mass spectrometry. Ketamine hydrochloride and xylazine were purchased from Vedco, Inc. (St. Joseph, MO) and Akorn, Inc. (Decatur, IL), respectively. Gadolinium diethylenetriaminepentaacetate (Gd-DTPA) contrast agent used for MRI was obtained from Bayer Healthcare (Leverkusen, Germany). All other reagents and solvents were purchased from Sigma Aldrich Chemical Company (St. Louis, MO).

Synthesis of Camptothecin-Glutamate (CPT-Glu) Conjugate

CPT was conjugated to L-Glu via an ester bond between the alcohol functional group at C20 (20-OH group) on CPT and the alpha-carboxylic acid group of the L-glutamic acid (Glu) using previous methods with minor modifications (Figure 1).¹⁵ A mixture of suspensions of CPT (0.10 g, 0.288 mmol), scandium triflate (0.085 g, 0.173 mmol), N-Boc-L-Glu(OtBu)-OH (0.524 g, 1.728 mmol), and N, N-dimethylaminopyridine (0.11 g, 0.864 mmol) in 5.0 mL anhydrous dimethylformamide (DMF) was cooled to -8°C in salt water ice bath. Then, 1,3-diisopropylcarbodiimide (0.142 mL, 0.907 mmol) was added slowly into the reaction mixture, stirred at -8°C for 30 min, allowed to warm to room temperature, and reacted for 1 h. The reaction mixture was treated with water (50 mL) and extracted with 100 mL dichloromethane. The organic extract was washed sequentially with 0.1 M HCl (200 mL) and 0.1 M NaHCO₃ (200 mL), followed by water extraction. The organic layer was dried with sodium sulfate followed by evaporation under reduced pressure. The CPT-N-Boc-L-Glu(OtBu) was treated with a solution of dichloromethane-trifluoroacetic acid (1:1, 2 mL) and stirred at room temperature for 1 h. The solvent was removed under reduced pressure and the resulting residue was purified by reverse-phased HPLC using a C₁₈ semi-preparative

column. The fractions containing the desired product were pooled and concentrated followed by lyophilization to give 0.90 g of CPT-Glu conjugate (Figure 1). The mass spectrometry result produced a dominant peak at $m/z = 478.15$, which corresponds to the calculated molecular weight of CPT-Glu. The CPT-Glu was dissolved in DMSO- d_6 and analyzed using the Bruker Advance AV-III 500 NMR spectrometer. 1H -NMR (DMSO- d_6): δ 12.45 (s, 1H), 8.73 (s, 1H), 8.47 (s, 2H), 8.15 (d, 2H), 7.88 (m, 1H), 7.74 (t, 1H), 7.27 (s, 1H), 5.57 (s, 2H), 5.33 (s, 2H), 4.38 – 4.5 (s, 1H), 2.62 (m, 1H), 2.10 – 2.30 (m, 4H), 0.90 (t, 3H).

LC-MS/MS Method Development

Preparation of Stock and Standard Solutions—Stock solutions (1 mg/mL) of CPT, CPT-Glu, and SN-38 were dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C . Working solutions of CPT and CPT-Glu were prepared prior to experiments by diluting the stock solutions with acetonitrile:water (v/v 1:1). Final concentrations of CPT and CPT-Glu for calibration standards were in the range between 5 and 500 ng/mL. SN-38 was used as an internal standard (IS). Calibration curves for both CPT and CPT-Glu were constructed by plotting the ratio of the peak area of the analyte and IS at each concentration. These standards were used to determine the total concentration of CPT and CPT-Glu delivered to the brain of each rat. Working standards used for extraction recovery experiments were prepared by diluting stocks solutions in DMSO and phosphate buffer, pH 3.0 (1:1 v/v) before spiking in tissue homogenate.

Extraction Recovery from Brain Tissue Homogenate—Extraction recovery of CPT and CPT-Glu from brain tissue homogenate was determined as the ratio between the amount of analyte extracted from spiked brain homogenate and the amount of analyte extracted from spiked blank medium. Each analyte was evaluated at 50, 250, and 500 ng/mL concentrations. The brain of an untreated rat (blank) was homogenized, and 190 μL aliquots of tissue homogenate were spiked with 10 μL of working standards for CPT and CPT-Glu. Each of the working standards contained IS at a concentration of 25 ng/mL. Then, the mixture was vortexed for 1 min followed by addition of a mixture of phosphate buffer at pH 3 and acetonitrile (v/v 1:6, 1 mL) into the homogenate. The homogenate was vigorously vortexed for 1 min followed by centrifugation at 12000 rpm. The supernatant was isolated, transferred to a clean tube, and evaporated to dryness under nitrogen at 40°C . The dry extract was reconstituted in 300 μL of acetonitrile:water containing 0.1% formic acid (v/v 1:1), vortexed for 30 sec, and centrifuged for 5 min at 12000 rpm to remove additional precipitated proteins.

Extraction of Drug from Brain Tissue after in situ Brain Perfusion—A mixture of phosphate buffer at pH 3.0 and 0.30 M phosphoric acid (v/v 1:1) was added to each whole brain tissue. The mixture was homogenized using a PowerGen 700 tissue homogenizer. Aliquots (190 μL) from rat brain homogenate were spiked with 10 μL IS (25 ng/mL) and vortexed. The samples were then extracted with 1.0 mL of 0.30 M phosphoric acid and acetonitrile (v/v 1:6) and vortexed vigorously for 1 min. Each homogenate was centrifuged at 12000 rpm for 5 min. The resulting supernatant was isolated and evaporated to dryness under nitrogen at 40°C . The residue was resuspended in 300 μL of acetonitrile:water with 0.1 % formic acid (v/v 1:1). Samples were then vortexed and centrifuged (12000 rpm for 5

min) to remove additional precipitated proteins. The sample (5 μ L) was injected into the LC-MS/MS.

Instrumentation and Chromatographic Conditions for LC-MS/MS—

Chromatography of each sample was performed on an Acquity UPLC system (Water Corp., Milford, MA) with a temperature-controlled autosampler set at 8°C. Separation was performed at room temperature on a Luna UPLC C₁₈ reversed-phase analytical column (2.1 mm $\times$ 50 mm, 5 μ m particle size, 100 Å; Phenomenex, Inc., Torrance, CA). A C₁₈ guard cartridge (4 mm $\times$ 2 mm ID) was used to protect the main column. The mobile phases consisted of a binary gradient system composed of solvent A containing H₂O:acetonitrile:formic acid (98.92:1:0.08) and solvent B containing acetonitrile:H₂O:formic acid (98.92:1:0.08). Analytes were eluted using a gradient system of 15% solvent B (initial), 15–30% solvent B (2.5 min), 30–90% solvent B (1 min), 90–15% solvent B (0.5 min), and 15% solvent B (1 min). The sample injection volume was 5 μ L. The samples were eluted at a flow rate of 0.40 mL/min for a total run time of 5 min.

On-line MS detection was performed on a Quattro Ultima triple quadrupole mass analyzer (Micromass Ltd., Manchester, UK) equipped with an electrospray ionization (ESI) source coupled to the UPLC system. Data were acquired in multiple reaction monitoring (MRM) mode to monitor the characteristic pseudomolecular ion [MH]⁺ of the compounds. Nitrogen was used as the desolvation gas, and argon gas was used for collision-induced dissociation (CID). All analytes were fragmented with cone voltage and collision energy set at 35V and 28V, respectively. MRM chromatograms were quantified using MassLynx v 4.1 software (Micromass UK Ltd) by integration of peaks.

In situ Rat Brain Perfusion Model for Delivery of CPT-Glu and Gd-DTPA Across the BBB

All protocols involving the use of animals, including *in situ* rat brain perfusion studies, were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Kansas. The *in situ* rat brain perfusion studies were done on adult male Sprague-Dawley rats weighing 250–350 g following similar methods described in previous studies.¹⁰ Brain delivery of CPT-Glu was done in the presence and absence of 1.0 mM HAV6 peptide. CPT-Glu (0.021 mmol/kg) was perfused in 10 mL of bicarbonate buffer (pH 7.4) containing 4.2 mM KCl, 1.5 mM CaCl₂, 0.9 mM MgCl₂, 128 mM NaCl, 2.4 mM NaH₂PO₄, and 24 mM NaHCO₃. Prior to the experiment, the perfusate was supplemented with D-glucose (6 mM) followed by filtration and oxygenation upon incubation under 95% O₂ and 5% CO₂ at 37°C. The rats were anaesthetized with a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg) delivered intraperitoneally. Then, the left common carotid artery (LCCA) was cannulated with a polyethylene catheter (PE 50) containing heparinized saline (100 IU/mL). The left pteryopalatine, occipital, and superior thyroid arteries were ligated using surgical thread. A heat lamp was used to maintain the animal's body temperature during the experiment.

Immediately after performing a heart-cut on the anesthetized rat, the LCCA was washed with saline delivered from a syringe pump (Model 341 B, Sage Instruments) at 5 mL/min for 10 sec. The brains were then perfused with 10 mL of 1.0 mM HAV6 in HCO₃⁻ buffer (pH 7.4) with 0.5 % Tween-20 at 5 mL/min. This was followed by perfusion of 10 mL of HAV6

(1.0 mM) and CPT-Glu (0.021 mmol/kg) in HCO_3^- buffer (pH 7.4) with 0.5 % Tween-20 at the same flow rate. Finally, a 10-sec post-perfusion wash with saline solution was delivered. The experiment was terminated by animal decapitation followed removal of the brain tissue. The whole brain was immediately stored in -80°C until further use. A similar study was done using only CPT-Glu as a control. The brains were first perfused with 10 mL of HCO_3^- buffer (pH 7.4) with 0.5 % Tween-20 at 5 mL/min. Then, 10 mL of CPT-Glu (0.021 mmol/kg) in HCO_3^- buffer (pH 7.4) with 0.5 % Tween-20 were subsequently perfused at the same flow rate. The samples were extracted and analyzed using LC-MS/MS as described above.

Similar to *in situ* brain perfusion of CPT-Glu, perfusion of Gd-DTPA in the presence and absence of HAV6 peptide was carried out followed by quantification of Gd-DTPA brain deposition using MRI. Briefly, the brains were perfused with 10 mL of 1.0 mM HAV6 in HCO_3^- buffer at pH 7.4 with 0.5 % Tween-20 at 5 mL/min. Then, a perfusion of 10 mL 0.6 mmol/kg Gd-DTPA (Magnevist, Berlex labs, NJ) in the presence of 1.0 mM HAV6 peptide in HCO_3^- buffer at pH 7.4 with 0.5 % Tween-20 was carried out at a flow rate of 5 mL/min. A 10-sec post-perfusion wash was delivered using saline solution. The amount of Gd-DTPA that penetrates across the BBB was determined from sacrificed animals at room temperature using a quantitative T_1 mapping MRI technique. Ex vivo MRI was performed using a quadrature volume coil in a 9.4 Tesla horizontal bore MR system equipped with a Agilent INOVA console (Agilent, Palo Alto, CA) and a 12-cm gradient insert (400 mT/m, 250 μs ; Magnex Scientific, Abingdon, UK). T_1 mapping was performed using a modified Look-Locker sequence for multi-slice and multiple phase encodings per inversion pulse ($\text{TR}/\text{TE} = 4/2$ ms, $\text{FOV} = 3$ cm, slice thickness = 1 mm, matrix size = 128×128 , flip angle = 20° , 22 inversion times from 40 – 5470 ms, 2 phasing encoding steps per inversion pulse, acquisition time = 8.5 min) and T_1 maps were generated using a program written in IDL (RSI, CO).¹⁶ $R_1 (= 1/T_1)$ values were obtained from regions of interest (ROI) placed in olfactory bulbs, cortex, striatum, hippocampus, cerebellum, spinal cord, ventral, deep-rostral (mostly hypothalamus and pallidum), and deep-caudal (mostly midbrain) regions. The experiments were performed with $n = 4$ for each experimental group (control, vehicle + Gd-DTPA, and HAV peptide + Gd-DTPA).

In Vivo Brain Delivery of Gd-DTPA with HAV6 Peptide as Monitored by MRI

The effect of HAV6 peptide in *in-vivo* brain delivery of Gd-DTPA was evaluated with *i.v.* administration of Gd-DTPA using T_1 -weighted MRI. Briefly, Sprague-Dawley rats were anesthetized using 3% isoflurane initially followed by 1–2% isoflurane in a gas mixture of air and oxygen at a ratio of 1:1. Two separate *i.v.* catheters (Insys Autogard, 22 GA, 0.9×25 mm, Becton Dickinson, Sparks, MD) were inserted into two separate tail veins and secured with tape. One of the *i.v.* catheters was used for infusion of the Gd-DTPA contrast agent and the other was used for infusion of either the vehicle control or the HAV6 peptide.

The animal was placed in the magnet in a prone position using an acrylic sled with its head held steady by two ear bars and a bite bar. The animal's body temperature was maintained at 37°C using a blanket with warm water circulation; its body temperature was monitored using a rectal temperature sensor (Cole-Palmer, Vernon Hills, IL). The animal's respiratory

rate was monitored using a pneumatic pillow sensor (SA instruments, Stony Brook, NY). A quadrature RF surface coil with two geometrically decoupled loops was used to transmit/receive MR signals at 400 MHz. T₁-weighted MR images were acquired using multi-slice spin-echo sequence (TR/TE = 240/10.5 ms, FOV = 3 cm, slice thickness = 2 mm, matrix size = 192 × 192, number of averages = 4, scan time per time point = 3 min).

Prior to infusion of the test compounds, baseline MR scans of the brain for the first 6 min (2 time points) were collected for each rat and this allowed each animal to serve as its own control. After the second time point, the contrast agent, Gd-DTPA (0.6 mmol/kg), was administered intravenously by an initial bolus followed by a slow infusion *via* the tail vein catheter to establish a rapid rise and subsequent plateau of plasma Gd-DTPA concentration during the study. After 10th time point (~30 min), either HAV6 peptide (0.019 mmol/kg) or vehicle was administered intravenously through the second tail vein catheter by a slow infusion for 1.0 min. MR scans were acquired immediately after the administration of the peptide or vehicle for 45 min. Time courses of T₁-weighted MRI signals were obtained from regions of interest (ROI) placed in sagittal sinus, muscle, olfactory bulb, deep rostral region (mostly hypothalamus and pallidum), deep caudal region (mostly midbrain), striatum, cortex, hippocampus, cerebellum, and spinal cord regions. To compare Gd-DTPA delivery to the brain between control and HAV6 peptide groups, signal intensities of T₁-weighted MR images were first normalized using the average signals of time points 1 and 2 (before contrast agent administration) and again with the averaged of time points 9 and 10 (prior to peptide infusion). Relative changes of T₁-weighted signal intensities following Gd-DTPA administration are proportional to the Gd-DTPA concentration in the ROIs. This normalization scheme removed the effect of variations among animals in clearance of Gd-DTPA. Areas under the curve (AUCs) were calculated by integrating the normalized time courses over time for each region of interest.

Statistical Analysis

Statistical analysis was done using one-way ANOVA with student-Newman-Keul post hoc tests to compare the difference in the deposition of total amount of CPT in the brain when CPT-Glu was administered in combination with HAV6 peptide and when CPT-Glu was administered alone. For MRI studies, AUC values obtained from animals receiving both Gd-DTPA and HAV6 peptide were compared to those obtained from the control groups. Drastic changes in the MRI signal represented significant perturbation of the BBB. A *p*-value of less than 0.05 was used as a criterion for statistical significance.

RESULTS

Synthesis of CPT-Glu Conjugate to Improve Solubility

The synthesis of CPT-Glu involved a two-step reaction in DMF by forming an ester bond between the α-COOH of Boc-L-Glu(OtBu)-OH and the 20-OH of camptothecin (Figure 1). The carboxylic acid group of Boc-L-Glu(OtBu)-OH was activated by 1,3-diisopropylcarbodiimide (DIPC) and reacted with CPT in the presence of DMAP and scandium triflate as catalysts. This conjugation reaction generated about 90% yield of the desired product; however, this reaction produced isomerization at the α-carbon of the Glu

residue, as previously observed.¹⁵ The tert-butyl ester and Boc-protecting groups were cleaved by 50% TFA in DCM to give the crude CPT-Glu. The crude CPT-Glu was purified using a reversed-phase C₁₈ column to produce a pure conjugate; mass spectrometry ESI-MS and ¹H-NMR confirmed the identity of the final pure product.

Detection and Analysis of CPT, CPT-Glu, and SN-38 by LC-MS/MS

Standard solutions of CPT, CPT-Glu, and SN-38 (IS) were first analyzed using MS and MS/MS to obtain their precursor and product ions, respectively. ESI full scan spectra produced dominant peaks of MH⁺ at m/z 349.1 for CPT, m/z 478.1 for CPT-Glu, and m/z 393.1 for SN-38. LC-MS/MS collision induced dissociation of each MH⁺ ion to produce major fragments at m/z 305.2 for CPT (Figure 2A) and m/z 331.0 for CPT-Glu (Figure 2B). Product ions from both CPT and SN-38 were derived from the loss of CO₂ (–44 u) from the lactone ring (Figure 2A).¹⁷ The CPT fragment of CPT-Glu was generated via the loss of the Glu residue (–147 u) upon ester bond cleavage (Figure 2B). The precursor and product ions obtained from MS/MS spectra were used to generate transitions for their use in MRM. The transition pairs used in MRM detection for each analyte were m/z 349.1 >> 305.2 for CPT, m/z 478.1 >> 331.0 for CPT-Glu, and m/z 393.1 >> 349.1 for SN-38 as IS. Figure 3 represents MRM chromatograms of rat brain tissue before and after the *in situ* brain perfusion with CPT-Glu. Analysis of brain tissues of untreated rats showed that there was no endogenous interference at the retention times of CPT, CPT-Glu, and SN-38 (data not shown). UPLC chromatography of CPT-Glu resulted in the elution of two peaks at 1.93 and 2.04 min (Figure 3A) with the same precursor/product transition, which is interpreted as the presence of a diastereomer likely generated during the esterification reaction. Both peaks were summed to represent the total amount of CPT-Glu detected. CPT-Glu was hydrolyzed by esterase enzymes to produce CPT in the brain (Figure 3B). The internal standard (IS) spiked into tissue homogenate after extraction was successfully separated from other analytes (Figure 3C).

Extraction Efficiency of CPT and CPT-Glu

To quantitatively determine the total amount of CPT delivered into the brain, extraction efficiency was determined for CPT and CPT-Glu from brain homogenates using previously published methods with minor modifications.¹⁸ The extraction recoveries for CPT and CPT-Glu were determined by comparing the ratio of a peak area of extracted analyte previously spiked into brain homogenates to a peak area of analyte spiked into extracted blank solutions. The recovery values from extracted brain were found to be 86–92% and 95–101% for CPT and CPT-Glu, respectively. These values are ideal for recovery of drugs from tissues where analysis can be hindered due to a high degree of protein-drug binding.

Calibration curves for CPT and CPT-Glu were constructed in biological matrices (brain and plasma) to determine the limit of detection (LOD), limit of quantification (LOQ), linearity, and range. The standards for CPT and CPT-Glu were around 1–500 ng/mL and 50–500 ng/mL, respectively. The calculated LOD and LOQ for CPT in biological matrices were found to be 2.4 and 7.9 ng/mL, respectively. CPT-Glu provided slightly higher values of LOD and LOQ, approximately 17 and 57 ng/mL, respectively. Both analytes showed good linear response with regression values of 0.99. Assay results from quality control samples

further demonstrated that this LC-MS/MS method had acceptable precision and accuracy. The coefficients of variation for inter-day and intra-day samples were found to be less than 10% as shown in Table 1, suggesting that the method was robust and could be used for analysis of samples extracted from brain homogenates.

Enhancing Brain Delivery of CPT-Glu and Gd-DTPA in the *In situ* Brain Perfusion Model

The activity of HAV6 peptide in improving paracellular transport of CPT-Glu across the BBB was evaluated using the *in situ* rat brain perfusion model. The rat brain was first perfused with a 10 mL solution containing 1.0 mM HAV6 peptide and then with a 10 mL solution of a mixture of CPT-Glu (0.021 mmol/kg) and 1.0 mM HAV6 peptide. As a control, the rat brain was perfused with 10 mL of vehicle followed by a 10 mL solution of CPT-Glu (0.021 mmol/kg). A flow rate of 5 mL/min was chosen because the hydrostatic pressure at this flow rate was high enough to ensure brain regional flow rates similar to those in physiological conditions without compromising the integrity of the tight junction.^{19, 20} The results showed that HAV6 peptide significantly enhanced brain delivery of CPT-Glu compared to control ($p < 0.05$) (Figure 4). Because some of the conjugate was converted to CPT by esterase in the brain, both CPT-Glu and CPT were used to calculate the total amount of transported CPT. The total amount of delivered CPT-Glu in the presence of HAV6 was 2058 ± 428 ng of CPT per g of rat brain while the total amount of delivered CPT-Glu in vehicle was 1282 ± 110 ng of CPT per g of rat brain.

The second study was carried out to evaluate the efficacy of HAV6 peptide to enhance brain delivery of Gd-DTPA using the *in situ* rat brain perfusion model, which was analyzed using MRI. In this case, Gd-DTPA (0.6 mmol/kg) and vehicle, Gd-DTPA and HAV6 peptide (1.0 mM), or vehicle only (control) was perfused into the brains followed by MRI scanning. The quantities of Gd-DTPA in different regions of the brain were expressed in R_1 values, whose differences from those without Gd-DTPA administration are proportional to the concentration of Gd-DTPA in the brain.²¹ Compared to vehicle only, rats perfused with Gd-DTPA and vehicle had increased R_1 values in cortex, brain-ventral, and deep-caudal, suggesting small amount extravasation of Gd-DTPA in the brain and/or residual intravascular Gd-DTPA in perfusate. Compared to Gd-DTPA and vehicle, rats treated with Gd-DTPA in the presence HAV6 showed more significantly increased R_1 values in the olfactory bulb (OB), hippocampus (HPC), cerebellum (CBLM), brain-ventral (BV), deep-rostral (DPRST), and deep-caudal (DPCDL) regions (Figure 5).

In Vivo Enhancement of Brain Delivery of Gd-DTPA upon i.v. Administration

In vivo modulation of the BBB using HAV6 peptide was evaluated in Sprague-Dawley rats by i.v. administration of Gd-DTPA (0.6 mmol/kg) in combination with vehicle or HAV6 peptide (0.019 mmol/kg). Ideally, Gd-DTPA administration should be limited to very low doses as possible. However, during our method development the contrast-to-noise ratio at 0.1 mmol/kg was low. The signal contrast at 0.6 mmol/kg was sufficient to provide visually appreciable MR signal changes and image contrasts and therefore we chose to increase it to 0.6 mmol/kg. Other studies have shown that single administration of Gd-DTPA at 1.0 mmol/kg in rats was well tolerated without any organ toxicity.²² Normalized T_1 -weighted MRI time courses from representative regions of interest are shown in Figure 6. The time

courses from the sagittal sinus area showed no significant difference in rats receiving Gd-DTPA with HAV6 peptide compared to those with vehicle, indicating similar systemic levels of Gd-DTPA (Figure 6B vs. 6E and 6C vs. 6F). In contrast, rats receiving Gd-DTPA + HAV6 peptide showed significantly higher T₁-weighted signal intensities than those receiving Gd-DTPA + vehicle. For instance, the MR images of the olfactory bulb in Figures 6A and D show that rats receiving Gd-DTPA plus HAV6 (Figure 6A) had significantly higher T₁-weighted signal intensities (as seen by more brighter areas) compared to the control group (Figure 6D); indicating higher accumulation of the contrast agent. T₁-weighted signal intensities were used to quantify the accumulation of Gd-DTPA in both groups. The time course profiles in various brain regions indicate a rapid alteration of the BBB following administration HAV6 peptide, as seen in the sharp increase of Gd-DTPA delivery within 3 min of injection of HAV6 peptide (Figure 7). In contrast, a steady decline without any increase in the time courses at various regions of the brain without HAV6 peptide reflects gradual clearance of Gd-DTPA from circulation. As shown in the sagittal sinus (Figure 7A), the muscle (Figure 7B), cortex (Figure 7C), the hippocampus (Figure 7D), olfactory bulb (Figure 7E), and deep rostral (Figure 7F) regions, we observed significantly higher overall accumulation of Gd-DTPA among the Gd-DTPA + HAV6 group compared to the control group; indicating enhanced brain delivery of Gd-DTPA in animals receiving HAV6 peptide. The area under the curves (AUCs) of various regions of the brains (Figure 8) shows significant enhanced accumulation of Gd-DTPA in the muscle (M), olfactory bulb (OB), deep rostral (DPRST), deep caudal (DPCDL), hippocampus (HPC), and cortex (CTX) regions of brains in animals received Gd-DTPA + HAV6 peptide compared to those received Gd-DTPA + vehicle.

DISCUSSION

The poor BBB penetration property of drugs and diagnostic agents is one of the major challenges in diagnosing and treating diseases affecting the CNS. One challenge to molecules in reaching the brain is due to recognition by an active efflux pump. Another concern is physicochemical properties of delivered molecules such as size, charge, and hydrophilicity, which prevent them from partitioning to cell membranes for crossing transcellular pathways of the BBB. In addition, tight junctions limit the paracellular permeation of molecules through the BBB. There are a few non-invasive strategies to improve brain delivery of drugs, which include the use of high osmotic mannitol²³⁻²⁵ and bradykinin receptor agonists such as CereportTM.²⁶ One potential drawback of using a high concentration of mannitol to disrupt the BBB is associated with a long recovery period that increases the risk of infection, neurotoxicity, and inflammation in the brain.²⁴ Although bradykinin agonists resulted in shorter recovery times, the clinical utility of these analogs was ineffective.^{23, 24} Thus, there is a need to find alternative methods to improve the delivery of drugs to the brain.

Our research focused on the use cadherin-derived peptides to increase the permeability of the BBB by inhibiting protein-protein interactions at the intercellular junctions in a more selective manner. In contrast to the long recovery periods required after the use of hypertonic mannitol solutions, we anticipate quick recovery and re-establishment of the integrity of the BBB following transient perturbation using cadherin peptides. Previous *in vivo* studies in

Balb/c mice have shown that the BBB opening produced by HAV6 was limited to less than 1 h,¹² indicating that the BBB integrity returned to normal within an hour. Here, the BBB modulatory activity of HAV6 was investigated in Sprague-Dawley rats to improve the delivery of CPT-Glu and Gd-DTPA into rat brains using an *in situ* brain perfusion model as well as *in vivo* brain delivery of Gd-DTPA via i.v. administration.

CPT is a naturally occurring alkaloid extracted from the *camptotheca acuminata* plant; it suppresses cancer proliferation by inhibiting topoisomerase-I enzyme activity required for cancer cell replication and transcription.²⁷⁻³⁰ CPT has been shown to have a wide spectrum of activities against human cancer malignancies such as lung, prostate, breast, colon, stomach, and ovarian carcinomas.³¹ Unfortunately, its utility to treat cancers, including brain tumors, has been limited by its unfavorable physicochemical properties. For example, CPT has very poor aqueous solubility (2.5 µg/mL), making it difficult to formulate.³⁰ The lactone group on CPT is stable below pH 5.5 and undergoes lactone hydrolysis to CPT-carboxylate at physiological conditions (pH 7.4) (Figure 1). CPT-carboxylate binds to human serum albumin (HSA), which causes lowering of the effective concentration of CPT in the bloodstream.²⁷ To solve the solubility problem, several derivatives of CPT such as topotecan (Hycamtin®) and irinotecan (Campto®) were developed. These derivatives are currently being used to treat patients with peripheral cancers such as various forms of ovarian, cervical, and lung cancers.^{27, 32} However, these current drugs have not been used to treat brain tumors because of their difficulty in crossing the BBB.

In this study, a CPT-Glu conjugate was synthesized to improve CPT solubility, and its brain delivery was investigated with the help of HAV6 peptide as a BBB modulator. Formation of CPT-Glu enhanced the water solubility more than 400-fold compared to that of CPT. Unfortunately, the formation of CPT-Glu produced diastereomers of GPT-Glu with racemization at the alpha-carbon of the Glu residue. However, both GPT-Glu diastereomers (Figure 3B) were successfully delivered into the brain using an *in situ* rat brain perfusion model, and CPT was also observed in the brain homogenate (Figure 3C). Because HAV6 peptide enhanced the paracellular transport of molecules, it was reasoned that the diastereomeric structures of CPT-Glu did not influence their penetration through the intercellular junctions of the BBB (Figure 4).

The acidic extraction method developed was efficient in extracting the conjugate (CPT-Glu) along with its hydrolyzed product CPT from brain tissue homogenates. After delivery, some of CPT-Glu molecules were converted to CPT via cleavage of the esterase bond. Ester formation at the 20-OH group has been shown previously to stabilize the lactone A ring at physiological pH, preventing the loss of activity associated with lactone ring opening.^{29, 33} Conjugation of the 20-OH group with different moieties has been shown to improve the physicochemical properties and pharmacokinetic profiles of CPT.^{29, 30, 34, 35} The propionate ester of 20-OH in CPT did not show any lactone ring hydrolysis when incubated for 4 h in phosphate-buffered saline solution containing 4% human plasma, while CPT alone was rapidly converted to the CPT-carboxylate form in 11 min (Figure 1).³⁶⁻³⁸ These results support the idea that formation of the ester increases the stability of the lactone ring in CPT.

The extraction and LC-MS/MS detection methods were successfully developed for quantification of delivered CPT-Glu and CPT in the brain. The LC-MS/MS method was developed to detect both CPT-Glu and CPT and for validation of the method. It started with the determination of product ions from CPT-Glu and CPT. Full scan spectra of all analytes were generated using electrospray ionization (ESI) positive ion mode in a solvent system consisting of 50:50 acetonitrile and water with addition of 0.1% formic acid (Figures 2 & 3). CPT, CPT-Glu, and SN-38 produced protonated molecular ion peaks. The SN-38 (IS) was spiked in all matrices prior to introduction to LC-MS/MS as a control for HPLC injection and ionization variability. When CPT, CPT-Glu, and SN-38 were subjected to collision-induced dissociation (CID), the energy applied fragmented the lactone ring, removing the CO₂ residue from both CPT and SN-38 molecules and producing a stable abundant product ion at m/z 305.2 and 349.1, respectively. CPT-Glu produced a major product at m/z 331.1 when analyzed under the same conditions (Figure 2B). This fragment occurred from cleavage of the ester bond at the 20-OH position of the CPT lactone ring. Comparison of the fragmentation pattern of CPT-Glu (m/z 478.1 $\gg$ m/z 331.1) to that of CPT (m/z 349.1 $\gg$ m/z 305.2) showed that the fragmentation of CPT-Glu did not involve the lactone ring. This indicates that the addition of the L-Glu amino acid residue to CPT offers some lactone ring stability as previously mentioned with CPT propionate ester.

For method validation, quality control (QC) samples were used to check for accuracy, precision, sensitivity, linearity of calibration curves, matrix effects, recovery, stability, and reproducibility according to the FDA guidance for bioanalytical method validation over a concentration range of 0–500 ng/mL.³⁹ CPT and CPT-Glu were quantitatively determined from peak areas calculated after the SRM scanning (Table 1). CPT-Glu had slightly higher LOQ and LOD of 57 ng/mL and 17 ng/mL, respectively. All of our QC samples were highly reproducible and had coefficients of variation between 0.99% and 14.17%. This indicates that this method is precise and accurate. Good linearity was observed over the studied concentration range, with $R^2 = 0.9$ or better. The extraction recoveries of CPT and CPT-Glu from brain tissue homogenates were excellent, with more than 86% efficiency over concentrations ranging from 1–500 ng/mL. Acetonitrile precipitation was used to remove interfering endogenous proteins as well as take advantage of the high solubility of CPT and CPT-Glu in organic solvents. Phosphate buffer, pH 3.0, was added to the extraction procedure to ensure determination of total amount of drug delivered in lactone form. All of the above indicate that our LC-MS/MS method was robust and suitable for quantification of CPT and CPT-Glu after the *in situ* brain perfusion.

Results from the *in situ* rat brain perfusion studies showed a significantly higher brain deposition of CPT-Glu when it was delivered with HAV6 peptide (1.6-fold) compared to delivery to CPT-Glu with vehicle (Figure 4). This was the first result to show that HAV6 peptide enhanced the delivery of CPT-Glu into the brain as detected by mass spectrometry. Besides detecting CPT-Glu, mass spectrometry also detected CPT in the brain, indicating that CPT-Glu was converted to CPT in the brain after delivery. This result confirmed previous *in situ* rat brain perfusion studies that HAV6 enhanced brain delivery of ¹⁴C-mannitol and [³H(G)]-daunomycin.¹⁰⁻¹²

Another method to confirm the BBB modulatory activity of HAV6 peptide was to deliver an MRI contrast agent, Gd-DTPA, into rat brains using the *in situ* rat brain perfusion model and living rats. The advantage of the MRI method is that it can evaluate the delivery efficiency of Gd-DTPA in various parts of the brain and this can also be done in living animals. Although it is very likely the CPT-Glu and Gd-DTPA might cross the BBB differently, we anticipate both molecules would cross the BBB predominantly via the paracellular route. It is widely accepted that Gd-DTPA would cross the BBB *via* the paracellular route due to its hydrophilic nature. Similarly, conjugation of CPT to glutamic acid would make it more hydrophilic as demonstrated by increased solubility in water. In addition to the difficulties encountered by drugs in crossing the BBB, another major obstacle in the delivery of drugs to the brain is the inability of the drugs to diffuse and penetrate the brain far away to the target site in pharmacologically relevant quantities. The pathway taken by the drugs to reach the site of action is highly tortuous and narrow. Here, we wanted to take advantage of the capabilities of MRI to evaluate the enhancement in the delivery of Gd-DTPA to various regions of the brain following modulation of the BBB permeability using cadherin peptide. MRI can provide a quantitative assessment of amount of tracer molecules present in the extracellular space. Contrast agent molecules present in the extravascular space shorten T1 relaxation time of water signals surrounding the contrast agent molecules and amplify the detectability of the tracer molecules dramatically and enables detection of very low levels of tracers. We believe that increased T1 weighted MRI signals in various areas of the brain sufficiently demonstrate the entry of contrast agent molecules into the extravascular space due to increased BBB permeability via HAV6 peptide perturbation both *in situ* and *in vivo*. The data from the *in situ* brain perfusion studies indicated that the depositions of Gd-DTPA were significantly higher in highly vascularized parts of the brain such as olfactory bulb, hippocampus, cerebellum, brain ventral, deep rostral, and deep caudal regions. MRI studies in living rats showed the *in vivo* BBB modulatory activity of the HAV6 peptide to enhance brain delivery of Gd-DTPA in various regions of the brain including muscle, olfactory bulb, deep rostral, deep caudal, hippocampus, and cortex regions, which consistent with the *in situ* brain perfusion study. The effect of HAV6 peptide in modulating the BBB to deliver Gd-DTPA was immediate or as soon as 3 minutes. This finding was consistent with previous studies to enhance brain delivery of Gd-DTPA using HAV6 and ADTC5 peptides in Balb/c mice.^{11, 12}

In our previous studies, we observed that increase in BBB permeability did not show any increase in cerebral blood flow;¹² suggesting that the differences in the increase in BBB regional permeability could be attributed to the differences in vasculature in these regions. Targeting abnormally highly vascularized regions would be beneficial in the treatment of brain tumors particularly glioblastomas. In contrast to normal blood vessels, tumor vasculature in glioblastomas are highly proliferative resulting in extensive vascularization which is critical for tumor progression and invasion.⁴⁰ One of the potential limitation of HAV6 peptide is that it also modulate E-cadherins in other organs (i.e., intestinal mucosa, lung and kidney). Thus, we are currently developing methods to improve selectivity of cadherin peptides to selectively modulate cadherins in the blood-brain barrier over cadherins in the other organs. In future, we hope to develop a more sensitive LC-MS/MS assay that would estimate regional enhancement of various molecules following transient BBB

disruption and correlate the results with the MRI data. Also, we hope to evaluate a number of molecules with different physicochemical properties and try to understand what properties are important for transport of drug molecules so that they can reach the target regions in therapeutically relevant quantities.

Cadherin peptides enhanced brain delivery of molecules in a global manner; in other words, the delivery of molecules was not specific to a certain part(s) of the brain. This is due to the potential mechanism of action of cadherin peptides in modulating the BBB. Cadherin peptides create a higher BBB permeability and paracellular transport of molecules because they disrupt cadherin-cadherin interactions in the intercellular junctions of the BBB. Our results showed that the quantities of brain deposition of Gd-DTPA were higher in parts of the brain with higher vascularization than section with lower vascularization regions (Figures 5 and 8). This is consistent with data from the in vivo studies in Balb/c mice, where three different cadherin peptides (i.e., HAV6, cHAVc3, and ADTC5) enhance the deposition of Gd-DTPA in the brain posterior region higher than the midbrain region.^{11, 12, 41} Furthermore, the deposition of Gd-DTPA in the midbrain region was higher than the anterior region.^{11, 12, 41} Thus, the quantities of brain depositions of delivered molecules correspond to the density of vascularization of each section of the brain. For a small molecule such as Gd-DTPA, cyclic cadherin peptides (i.e., cHAVc3 and ADTC5) allowed the brain delivery of Gd-DTPA when the peptides delivered 2 h earlier than Gd-DTPA.^{11, 41} However, no enhancement of Gd-DTPA brain delivery was observed when it was delivered 4 h after administration of cadherin cyclic peptides. This result indicates that cHAVc3 and ADTC5 open the BBB for about 2–4 h. In contrast, linear HAV6 peptide modulates the BBB in less than 1 h time frame.¹² For a large molecule such as IRdye-labeled 25 kDa polyethylene glycol (IRdye 800cw-PEG), both linear HAV6 and cyclic cHAVc3 peptides increase its brain delivery when administered together with peptide.^{12, 41} However, when the IRdye 800cw-PEG was delivered 1 h after administration of linear HAV6 or cyclic cHAVc3 peptides there was no enhancement of brain delivery.^{12, 41} In other words, large molecules could be only be delivered at the early state of the BBB modulation. This result suggests that the BBB size of opening by cadherin peptides is a time-dependent manner and the opening for small molecules has a longer time window than for large molecules. Our hypothesis is that cadherin peptides modulate the BBB by immediately generating both large and small openings in the intercellular junctions of the BBB; however, the large size openings collapse to small size openings as the time progresses.

The long-term opening of the BBB may cause unwanted side effects in the brain because the BBB opening could allow the penetration of unwanted molecules (i.e., proteins, peptides) and particles (i.e., cells) from the blood stream into the brain. Although in inflammatory situations the BBB is also leaky for allowing temporary infiltration of immune cells into the brain, the long-term opening could have unfavorable effect to the brain. The effects of repeated and long-term BBB opening by cadherin peptides have not yet been studied; thus, these effects will be studied in the future. It is interesting that cadherin peptides induce only a short period of the BBB opening for large molecules (i.e., IRdye 800cw-PEG); therefore, this is an advantage characteristic of cadherin peptide because it could prevent the delivery of large unnecessary or toxic molecules (e.g., protein) into the brain.

CONCLUSIONS

In conclusion, this study demonstrated that HAV6 enhanced the brain delivery of CPT-Glu and Gd-DTPA in an *in situ* rat brain perfusion assay compared to control. The HAV6 peptide also significantly improved brain deposition of Gd-DTPA in the *in vivo* study. The improved brain delivery of those molecules was attributed to increased permeability of the BBB upon intercellular junction modulation by HAV6 peptide. This modulation occurred in a matter of minutes after injection of HAV6 peptide. The developed LC-MS/MS was very sensitive and selective to identify and quantify CPT-Glu and CPT in the brain. The detection limits can be as low as 2.4 and 17 ng/mL for CPT and CPT-Glu, respectively. As shown from studies using MRI, the molecules were delivered more efficiently to the vascularized regions. In the future, HAV6 will be used to deliver CPT-Glu into the brains of animals (i.e., rat and mouse) with brain tumors and to deliver large molecules (e.g., peptides, proteins, nucleotides) into the brains of animals with brain diseases for treatments and diagnostic purposes.

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Figure 1. Structures of camptothecin (CPT), camptothecin-glutamate conjugate (CPT-Glu), and SN-38 as an internal standard as well as the synthetic scheme to make CPT-Glu. CPT is stable at pH below 5.5 and the lactone ring CPT-carboxylate at the physiological condition (pH 7.4).

Figure 2. Mass spectra for the daughter ions and MRM transitions for (A) CPT m/z 349.1 $\gg$ 305.2 and (B) CPT-Glu m/z 478.1 $\gg$ 331.0.

Figure 3. UPLC chromatograms of brain tissue homogenates for CPT, CPT-Glu, and SN-38. (A) CPT-Glu (1.93 and 2.04 min) extracted from brain tissue extract after *in situ* brain perfusion, (B) internal standard, SN-38 (25 ng/mL), spiked and extracted from tissue homogenate with retention time of 3.42 min and (C) hydrolyzed product CPT (3.59 min) detected in brain homogenate after perfusion studies.

Figure 4.

Total amount of equivalent CPT accumulation in the brain after *in situ* brain perfusion of CPT-Glu in the presence and absence of HAV6 peptide. Rat brains were perfused with CPT-Glu (0.021 mmol/kg) in the presence or absence of 1.0 mM HAV6 peptide at a flow rate of 5 mL/min. Data are represented as mean $\pm$ s.d. with $p < 0.05$.

Figure 5.

The MRI scans of the brain from *in situ* rat brain perfusion studies to compare the $R_1 = 1/T_1$ values of the brain regions of rats treated with Gd-DTPA (0.5 mmol/kg), Gd-DTPA (0.5 mmol/kg) with HAV6 peptide (1.0 mM), and vehicle. The differences in R_1 values were determined at various parts of the brain, including olfactory bulbs (OB), cortex (CTX), striatum (STR), hippocampus (HPC), cerebellum (CBLM), spinal cord (SPN), brain ventral (BV), deep rostral (DPRST), and deep caudal (DPCDL). Data are represented as mean $\pm$ SD. Statistical significance considered at $p < 0.05$, designated as (*) for vehicle vs. Gd-DTPA; (#) for vehicle vs. Gd-DTPA+HAV6, and (†) for Gd-DTPA vs. Gd-DTPA +HAV6.

Figure 6.

Representatives of MR images showing Gd-DTPA deposition at various brain regions following intravenous infusion of Gd-DTPA (0.6 mmol/kg) and HAV6 peptide (0.021 mmol/kg) or vehicle to induced disruptions of the BBB. Signal intensities of MR images at time point 16 were normalized with those at time point 1. MR images show that there was no significant difference in the levels of Gd-DTPA in the sagittal sinus region (arrow heads) between Gd-DTPA + HAV6 group (**B** and **C**) and that of Gd-DTPA alone (**E** and **F**). Gd-DTPA levels in the olfactory bulb were significantly higher in (**A**) Gd-DTPA + HAV6 group compared to (**D**) Gd-DTPA alone; suggesting that HAV6 improved the transport of Gd-DTPA across the BBB. Gray scale bar indicates normalized signal intensities ranges of 0–3.0.

Figure 0007

B

C

D

E

Figure 0008

F

Figure 0012

Figure 7.

Amount of Gd-DTPA in various regions of the brain over time to show kinetic deposition of Gd-DTPA following intravenous infusion of Gd-DTPA and HAV6 peptide or vehicle to induced disruptions of the BBB. At 0–6 min, the brain was scanned as the baseline. At 6 min, Gd-DTPA (0.6 mmol/kg) was delivered i.v. followed by observation until 10th time-point (~30 min). HAV6 peptide (0.021 mmol/kg) or vehicle was injected i.v. immediately after 10th time-point and the depositions of Gd-DTPA were monitored up to 45 min. Time courses of T₁-weighted MRI signals were normalized first with those before Gd-DTPA administration and then with those before HAV6 peptide administration. Normalized time courses are from (A) sagittal sinus, (B) muscle, (C) cortex, (D) hippocampus, (E) olfactory bulb, and (F) deep rostral. Data is represented as mean ± SD. (*) indicates significant differences between Gd-DTPA+HAV6 and Gd-DTPA+vehicle group with $p < 0.05$.

Figure 8. Area under curve (AUC) values showing deposition of Gd-DTPA in various regions of rat brain following intravenous infusion of Gd-DTPA+vehicle and Gd-DTPA+HAV6 peptide. SS = sagittal sinus; M = muscle; OB = olfactory bulb; DPRST = deep-rostral; DPCDL = deep-caudal; STR = striatum; HPC = hippocampus; CTX = cortex; CBLM = cerebellum; and SPN = spinal cord. Data are represented as mean $\pm$ SD. (*) indicates significant differences between groups Gd-DTPA+HAV6 and Gd-DTPA+vehicle with $p < 0.05$.

Table 1
Accuracy and precision for the analysis of CPT and CPT-Glu spiked and extracted from rat brain homogenate (n = 3, duplicates per day)

Analyte	Nominal Conc. (ng/mL)	Measured Conc. (ng/mL)	Intra-day cv (%)	Inter-day cv (%)	Relative error (%)
CPT	5	4.58	8.24	8.85	8.4
	50	48.03	2.52	4.67	3.95
	250	234.57	5.35	3.97	6.17
CPT-Glu	50	49.57	2.71	4.32	0.86
	100	98.26	1.67	5.98	1.75
	250	251.08	2.18	4.67	0.43