Journal of Pharmaceutical Sciences 105 (2016) 797–807

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutics, Drug Delivery and Pharmaceutical Technology

Comparison of Linear and Cyclic His-Ala-Val Peptides in Modulating the Blood-Brain Barrier Permeability: Impact on Delivery of Molecules to the Brain

Ahmed Alaofi [1], Ngoc On [2], Paul Kiptoo [1], Todd D. Williams [3], Donald W. Miller [2], Teruna J. Siahaan [1,*]

[1] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66047
[2] Department of Pharmacology and Therapeutics, University of Manitoba, Winnipeg, Manitoba, Canada
[3] Mass Spectrometry Laboratory, The University of Kansas, Lawrence, Kansas 66047

ARTICLE INFO

Article history:
Received 29 September 2015
Revised 29 October 2015
Accepted 3 November 2015

Keywords:
blood-brain barrier
BBB
brain delivery
cadherin peptides
HAV peptide
paracellular pathway
tight junction
adherens junction
BBB modulation

ABSTRACT

The aim of this study is to evaluate the effect of peptide cyclization on the blood-brain barrier (BBB) modulatory activity and plasma stability of His-Ala-Val peptides, which are derived from the extracellular 1 domain of human E-cadherin. The activities to modulate the intercellular junctions by linear HAV4 (Ac-SHAVAS-NH_2), cyclic cHAVc1 (Cyclo(1,8)Ac-CSHAVASC-NH_2), and cyclic cHAVc3 (Cyclo(1,6)Ac-CSHAVC-NH_2) were compared in *in vitro* and *in vivo* BBB models. Linear HAV4 and cyclic cHAVc1 have the same junction modulatory activities as assessed by *in vitro* MDCK monolayer model and *in situ* rat brain perfusion model. In contrast, cyclic cHAVc3 was more effective than linear HAV4 in modulating MDCK cell monolayers and in improving *in vivo* brain delivery of Gd-DTPA on i.v. administration in Balb/c mice. Cyclic cHAVc3 ($t_{1/2}$ = 12.95 h) has better plasma stability compared with linear HAV4 ($t_{1/2}$ = 2.4 h). The duration of the BBB modulation was longer using cHAVc3 (2-4 h) compared with HAV4 (<1 h). Both HAV4 and cHAVc3 peptides also enhanced the *in vivo* brain delivery of IRdye800cw-PEG (25 kDa) as detected by near IR imaging. The result showed that cyclic cHAVc3 peptide had better activity and plasma stability than linear HAV4 peptide.

Introduction

It is challenging to treat brain diseases such as Alzheimer and Parkinson and brain tumors because drugs used to treat these disorders have difficulty in crossing the blood-brain barrier (BBB).[1-3] The microvascular endothelial cells of the brain have tight junctions that limit paracellular diffusion,[3] and there are various efflux transport proteins (P-glycoprotein [P-gp], breast cancer resistance protein, multidrug resistance-associated proteins) and metabolic enzymes that reduce transcellular routes of entry to the brain.[4-6] To cross the BBB, the drug molecule must have optimal physicochemical properties; for example, many large molecules such as peptides and proteins cannot readily cross the BBB because of their size and hydrophilicity.[1,3,7] For example, nerve growth factor, and brain-derived neurotrophic factor have been investigated to treat neurodegenerative diseases.[8-13] Unfortunately, as with other proteins, the brain delivery of nerve growth factor and brain-derived neurotrophic factor is challenging. Therefore, there is a need for safe and effective methods to improve the delivery of proteins and peptides to the brain.

Modulation of the BBB paracellular pathway is one way to improve brain delivery of proteins and peptides. BBB modulation can be achieved in a variety of different ways; one example is the use of vasoactive agents (i.e., bradykinin, bradykinin analogs, histamine, lysophosphatidic acid) to disrupt the BBB.[14] However, these agents produce their effects through activation of receptors on the brain endothelial cells and can potentially cause rapid desensitization, pathologic changes in receptor density, and damage to the BBB.[14] Infusion of hyperosmolar mannitol (25% solution) has also been used to temporarily open the BBB tight junctions to allow both lower (i.e., methotrexate) and higher molecular weight (i.e., Evan blue-albumin) molecules to enter the brain.[15,16]

A more selective method to modulate the paracellular pathway of the BBB uses peptides to inhibit protein-protein interactions in the intercellular junctions of the BBB. Examples include inhibitory

* *Correspondence to:* Teruna J. Siahaan (Telephone: +1-785-864-7327; Fax: 785-864-5736).
E-mail address: siahaan@ku.edu (T.J. Siahaan).

http://dx.doi.org/10.1016/S0022-3549(15)00188-4
0022-3549/

Table 1
The Names, Sequences, and Molecular Weights of Cadherin Peptides

Peptide Name	Sequence	Molecular Weight[a] (Da)
HAV6	Ac-SHAVSS-NH_2	627.2
HAV4	Ac-SHAVAS-NH_2	611.3
cHAVc1	Ac-CSHAVASC-NH_2	815.3
cHAVc3	Ac-CSHAVC-NH_2	657.2
ADTC5	Ac-CDTPPVC-NH_2	772.2

[a]Molecular weight of peptide was determined by mass spectrometry.

peptides targeting claudin-1 (i.e., C1C2 peptide) and occludin (i.e., OCC1 and OCC2 peptides) that modulate tight junctions in various *in vitro* models of the BBB[17,18] and increase the brain delivery of the opioid receptor agonist [D-Ala,[2] N-MePhe,[4] Gly[5]-ol]-enkephalin (DAMGO).[19] In the case of C1C2 peptide, the mechanism of action involves relocalization of claudin-1 from the cell surface to cytosol, resulting in a long-lasting modulation of the BBB.[20,21] Our work has focused on cadherin peptides that modulate cadherin interactions in the adherens junctions of both endothelial and epithelial cells. The His-Ala-Val (HAV) and Ala-Asp-Thr (ADT) peptides derived from the extracellular 1 domain of E-cadherin have been shown to enhance delivery of ^{14}C-mannitol *in vitro* in Madin–Darby canine kidney (MDCK) cell monolayers.[22,23] Recently, both HAV6 (Table 1) and ADTC5 peptides have been shown to enhance the brain delivery of both small and large molecules into the brain of rats and mice.[24-26]

In this study, cyclic HAV peptides (cHAVc1 and cHAVc3) were designed to improve BBB modulatory activity compared with that of linear HAV4 peptide (Table 1). The hypothesis was that the increased backbone rigidity provided by cyclic HAV peptides would result in improved binding affinity for the extracellular domain of cadherin as well as improved plasma stability compared with that of the linear peptide. Therefore, cyclic HAV peptides (i.e., cHAVc1, cHAVc3) were synthesized by forming a disulfide bond between 2 cysteine residues added to the N- and C-termini of the original linear peptide (Table 1). The plasma stabilities of linear HAV4 and cyclic cHAVc3 peptides were determined in rat plasma, and the peptide degradation was detected and quantified by mass spectrometry. The adherens junction modulatory activity of cyclic cHAVc3 peptide was compared with that of linear HAV4 using *in vitro* MDCK cell monolayers, and the modulatory effects on BBB permeability were examined using the *in situ* rat brain perfusion model as well as in the *in vivo* Balb/c mouse model. The results indicated that cyclic cHAVc3 peptide has better BBB modulatory activity and plasma stability than does the linear HAV4 peptide.

Materials and Methods

Peptide Synthesis

Cyclic and linear HAV peptides were synthesized using solid-phase method with Fmoc chemistry in a perceptive pioneer peptide synthesizer as previously described.[22,23] HAV peptides were cleaved from resin and purified by reversed-phase HPLC using a C18 column. The disulfide bond in cyclic peptides was formed by bubbling air into a dilute solution of precursor linear peptides in ammonium bicarbonate buffer at pH 8.5. The identity of each peptide was determined by mass spectroscopy.

In Vitro *Peptide Modulatory Activity in MDCK Cell Monolayers*

Cell Culture

The *in vitro* modulatory activities of linear and cyclic HAV peptides were evaluated in MDCK cell monolayers, and this model was selected to evaluate the effect of cadherin peptides in modulating the intercellular junctions of the cell monolayers. The MDCK-II cells (cat. no. 00062107) were acquired from European Collection of Authenticated Cell Cultures (ECACC), Salisbury, UK, and were seeded into Corning flasks until they reached 80% confluency as a monolayer. Then, the cell monolayer was washed twice with phosphate-buffered saline (PBS) followed by treatment with trypsin–ethylenediaminetetraacetic acid solution (0.25% trypsin, 1.0-mM ethylenediaminetetraacetic acid in Hank's Balanced Salt Solution [HBSS]). The detached single cells were then resuspended, collected, and counted. They were then added into each well (75,000 cells/well) of a Transwell® plate (Permeable Supports, 0.4-μM polyester membrane, 12-well plates) and were incubated for 5-8 d. Trans-epithelial electrical resistance (TEER) values were measured before and at the day of the experiment to check the monolayer integrity.

Inhibition of Junction Resealing

The inhibition of junction-resealing in MDCK-II cell monolayers was used to compare the modulatory activity of linear and cyclic HAV peptides.[23] In this study, the changes in TEER values were followed using an EVOM™ voltohmmeter (World Precision Instruments) in the presence and absence of HAV peptides. Each experiment was started at TEER values of 280-320 Ω.cm^2 for the monolayers. After the cell monolayers were confluent, they were washed with HBSS solution containing 25-mM glucose, 2-mM $CaCl_2$, 0.75-mM $MgSO_4$, and 10-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The cell monolayers were incubated with HBSS for 1.5 h, and the TEER values were recorded. Then, the cell monolayers were washed and incubated with Ca^{2+}-free buffer to open the tight junctions. The intercellular junction opening caused a decrease in TEER values of 50%-60%. The cells were then incubated in Ca^{2+}-sufficient medium to reseal the intercellular junctions in the presence and absence of HAV peptides (1.0 mM) on the apical and basolateral sides. During the resealing of the intercellular junctions, the TEER values were recorded every hour for 6-8 h.

Direct Junction Modulation

The activities of HAV peptides were evaluated by directly modulating intact MDCK-II cell monolayers. In this assay, only Ca^{2+}-sufficient buffer was used. The Transwells were washed with HBSS buffer containing 25-mM glucose, 2-mM $CaCl_2$, 0.75-mM $MgSO_4$ and 10-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid. The MDCK cell monolayers were incubated with and without HAV peptides (1.0 mM) on the apical and basolateral sides followed by measurement of TEER values every hour for 6-8 h.

Peptide Stability in Rat Plasma

The rat plasma (lot. no. 19595; NaHeparin) used for plasma stability studies was purchased from Innovative Research, Inc. (Novi, MI). The plasma degradations of cyclic cHAVc3 and linear HAV4 were determined using liquid chromatography–tandem mass spectrometry, and the half-life of each peptide in plasma was calculated. Briefly, HAV4 or cHAVc3 peptide in 1.2 μL of DMSO was added into 200 μL of rat plasma to make the final concentration of DMSO 0.6%. Each peptide solution in rat plasma was incubated and agitated using an orbital shaker at 50 rpm at 37°C for up to 2-3 half-lives, which were 72 h for cyclic cHAVc3 and 8 h for linear HAV4. The peptides were extracted from plasma using liquid-liquid extraction. In this case, 201.2 μL of plasma containing peptide was added into a 1.0-mL solvent mixture of ACN:H_2O:EtOAc (6:1:1) to precipitate plasma proteins; this was followed by centrifugation at 17226*g* (12000 PRM) using Centrifuge-5415D (Eppendorf AG-22331 Hamburg, Germany). The supernatant was collected and