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N-Methyl Phenylalanine-Rich Peptides as Highly Versatile Blood−Brain Barrier Shuttles

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Here we studied the capacity of *N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, Cha-(*N*-MePhe)$_3$-CONH$_2$, and 2Nal-(*N*-MePhe)$_3$-CONH$_2$ to carry various drugs (cargos) in in vitro blood−brain barrier (BBB) models in order to determine the versatility of these peptides as BBB-shuttles for drug delivery to the brain. Using SPPS, the peptides were coupled to GABA, Nip, and ALA to examine their passive BBB permeation by means of PAMPA and their lipophilicity by IAMC. Unaided, these nonpermeating drugs alone did not cross the PAMPA barrier and the BBB passively; however, the peptides tested as potential BBB shuttles transferred them by passive transfer through the PAMPA phospholipid. The permeability of peptides that showed the highest permeability in PAMPA, and Ac-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ as the parent peptide was also examined in bovine brain microvessel endothelial cells (BBMECs). These peptide-based BBB shuttles open up the possibility to overcome the formidable obstacle of the BBB, thereby achieving drug delivery to the brain.

Introduction

Unlike many peripheral blood vessels, the endothelial cells of brain capillaries attach to each other to form tight junctions without fenestration, which prevents paracellular transport. Therefore, to gain access to the brain, compounds must cross this barrier, known as the blood−brain barrier (BBB[a]), via transcellular routes, including energy-dependent transport and passive diffusion.

Although novel neuropharmaceutical drugs are available to treat some brain disorders, the presence of the BBB impedes their reaching their site of action in the brain and consequently they are not effective.[1] In addition, the BBB can make the treatment of noncentral nervous system (CNS) diseases difficult. For example, the human immunodeficiency virus-1 (HIV-1) enters the brain through adsorptive-mediated endocytosis;[2,3] however, drugs for the treatment of the acquired immunodeficiency syndrome (AIDS) do not cross the BBB,[4,5] therefore the brain can act as an HIV-1 reservoir and, despite of drug availability, treatment is complicated. Thus new approaches to deliver drugs to the brain should not be limited only to the treatment of the CNS diseases.

There are two methods, among others, to convert hydrophilic drugs, which do not enter the brain, into drugs with capacity to cross the BBB. The first involves the reformulation of drug structures so that the resulting molecules become substrates for the endogenous BBB transporters (i.e., L-dopa);[6] however, knowledge of the BBB endogenous transport systems is required and, in addition, this alteration should not influence on the biological effect of the drugs when inside the brain. The second method comprises a peptide vector-mediated strategy in which nontransportable drugs are linked to peptides that have the capacity to cross the BBB. The latter was used by Rousselle et al. and Temsamani et al. to improve the brain uptake of several drugs, such as doxorubicin,[7] penicillin,[8] enkephaline analogue dalargin,[9] paclitaxel,[10] and morphine-6-glucuronide.[11] In some cases, this conjugation even increased drug solubility and bypassed the P-glycoprotein,[7,10] therefore discarding the need for solubilizers, which could cause side effects, and for P-gp inhibitors, which limit the clinical application of drugs. Schwarze et al.[12] demonstrated that an 11-amino acid peptide from TAT protein delivered β-galactosidase (β-Gal) protein to several tissues, including the brain.

We have recently reported that small cyclic *N*-methylated and *N*-MePhe-rich peptides could act as BBB shuttles and transport L-dopa and baicalin through the PAMPA membrane, an in vitro model of the BBB.[13,14] PAMPA is a noncell-based assay with an artificial membrane that was introduced by Kansy[15] for the prediction of gastrointestinal tract absorption. During the past decade, PAMPA, which presents only nonsaturable transmembrane diffusion, has been modified to be used as an in vitro model to study BBB and skin permeation[16−18] to predict the cellular activity of several hepatitis C virus protease inhibitors[19] and the passive elimination rate constant in fish.[20] It has also been applied to studies to determine the mechanism of drugs permeation,[21] the partition

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[a]Abbreviations: ACH, α-cyano-4-hydroxy-cinnamic acid; BBB, blood−brain barrier; CNS, central nervous system; Da, dalton; DBU, 1,8-diazabicyclo[5.4.0]-undec-7-en; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOAt, 7-aza-1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; HPLC-MS, high performance liquid chromatography mass spectrometry; HRMS, high resolutiom mass spectrometry; IAMC, immobilized artificial membrane chromatography; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MeCN, acetonitrile; MTBD, 7-methyl-1,5,7-triazabicyclo[4.4.0]dec-5-ene; *o*-NBS, *o*-nitrobenzenesulfonyl chloride; PAMPA, parallel artificial membrane permeability assay; PyBOP, benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; t_R, retention time.

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coefficient between water and different solvents,[22] and the permeation of compounds that can not be determined by cell-based assays because of compound instability.[23] PAMPA is a rapid, uncomplicated, and low-priced method compared to cell-based assays. Di et al.[24] illustrated that PAMPA correlated with in situ brain diffusion remarkably better than MDR1-MDCKII for a broad range of CNS drugs with diverse physicochemical properties available on the market. The transport of compounds across in vitro membranes is limited by a barrier formed by the membrane and the two unstirred water layers (UWL), one on each side of the membrane. UWL, which is <1 μm in the BBB, is the rate limiting factor for the transport of highly lipophilic molecules. If its effect is not corrected with a suitable protocol, the resulting permeability shows only properties of water instead of membrane. Shaking the solution can reduce the thickness of UWL.[25−28] Thus, to better mimic the BBB in vivo, we performed all PAMPAs by placing the PAMPA plate on an orbital shaker at 100 rpm. Given the properties of PAMPA described above and that our peptides concern only passive transport, PAMPA was used as a high-throughput in vitro assay to assess peptide permeability. In all the experiments, propranolol and carbamazepine, two CNS drugs, were used as positive controls.

Here, we studied the efficiency of three *N*-MePhe-rich peptides, *N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, Cha-(*N*-MePhe)$_3$-CONH$_2$, and 2Nal-(*N*-MePhe)$_3$-CONH$_2$ (Figure 1A), to carry a series of structurally unrelated drugs in PAMPA, IAMC, and BBMECs with the aim to test the capacity of these peptides as versatile BBB shuttles for drug delivery to the brain. For this purpose, 4-aminobutanoic acid (GABA), nipecotic acid (Nip), and 5-aminolevulinic acid (ALA) (Figure 1B) were coupled to the peptides and evaluated by PAMPA, IAMC, and BBMECs. These peptides carried a variety of small drugs (molecular weight <200 Da), which cover a wide range of structures such as linear, cyclic, and aromatic[14] compounds, across the PAMPA barrier and can therefore be considered versatile BBB shuttles for small neurodrugs. Although differences in the permeability of each cargo-peptide construct were detected, the peptide *N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ showed the best shuttle performance for all cargos while the peptide 2Nal-(*N*-MePhe)$_3$-CONH$_2$ was the least effective. These findings indicate that these peptides can be envisaged as promising BBB shuttle candidates for drug delivery to the brain.

Results and Discussion

High-Throughput Liquid Chromatography/Mass Spectrometry (LC/MS) Method for PAMPA Permeability Measurement. First, we determined the PAMPA permeability of three selected cargos (GABA, Nip, and ALA). However, these cargos lack UV-absorbing chromophore to be quantified by UV without derivatization, thus HPLC-UV cannot be used to measure them in PAMPA donor or acceptor wells. The use of MS to overcome this problem has been proposed in the literature.[29,30] In our case, first we used the areas underneath ion current profiles from HPLC-MS measurements to generate two calibration curves for each of the selected cargos, one as acceptor curve ranging from 0.2 to 10 μM and the other as donor curve ranging from 20 to 200 μM consisting of seven and six points, respectively. All the curves were linear and showed excellent coefficient correlation (Figure 2). The cargos were then evaluated by PAMPA and analyzed by HPLC-MS. None of the cargos permeated across the

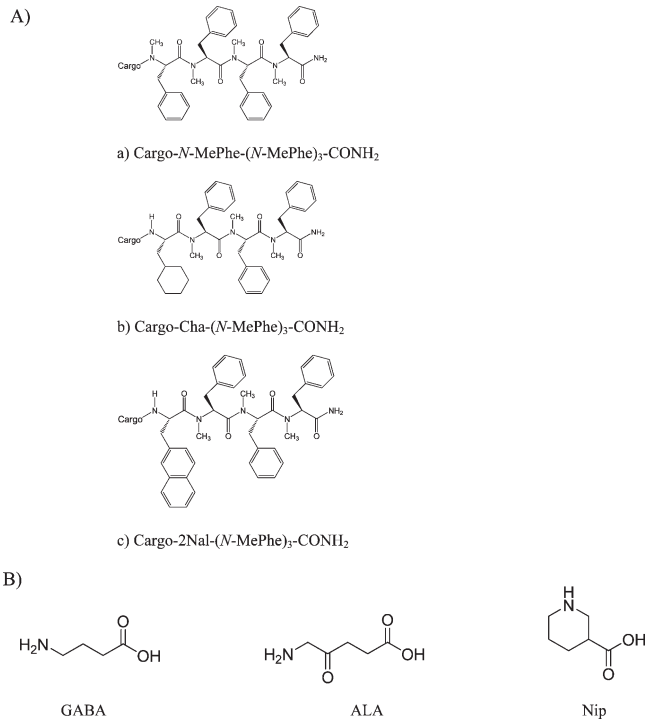

Figure 1. (A) Structures of three cargo-*N*-MePhe-rich peptides (BBB-shuttles). (B) Structures of selected cargos GABA, ALA, and Nip.

PAMPA phospholipid barrier (Table 1); however, the amount of compounds that remained in the PAMPA membrane (membrane retention, %*R*) differed between cargos. This difference can be directly related to the different number of H-bonds that these compounds form with water. Nip can make four H-bonds,[31] and therefore it requires less desolvation energy to move from water into a phospholipid environment. Another explanation is that Nip can be considered a GABA or ALA analogue with less flexibility and therefore it shows stronger attachment or greater retention. This hypothesis is in line with an earlier study by Mahar et al.[32] reporting that CNS drugs have reduced flexibility compared to non-CNS drugs. While ALA makes one H-bond more than GABA (6 vs 5), its retention was unexpectedly greater in the PAMPA membrane. This observation could be attributed to the fact that the strength of H-bonds are not equal and they do not require the same energy to be disolvated and moved from water into membrane, as reported previously by Barbara et al.[33] An alternative explanation would be because the additional carbonyl group in ALA structure confers this molecule a greater tendency to attach to the membrane, as reported previously by Fischer et al.[34] These authors demonstrated that a difference of only one C atom between two structurally similar molecules, berberine and chelerythrine, causes a very large difference in their permeability in PAMPA. Using PAMPA and MS methodology, here we show for the first time that GABA, Nip, and ALA do not cross artificial membranes by passive diffusion.

Application of *N*-MePhe-Rich Peptides as BBB-Shuttles. In a preliminary study,[14] we previously showed that the *N*-MePhe-rich peptides *N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, Cha-(*N*-MePhe)$_3$-CONH$_2$, and 2Nal-(*N*-MePhe)$_3$-CONH$_2$ shuttled L-dopa across the artificial membrane. To explore the capacity of the peptides to carry another drug, GABA was chosen as a structurally diverse drug from L-dopa.

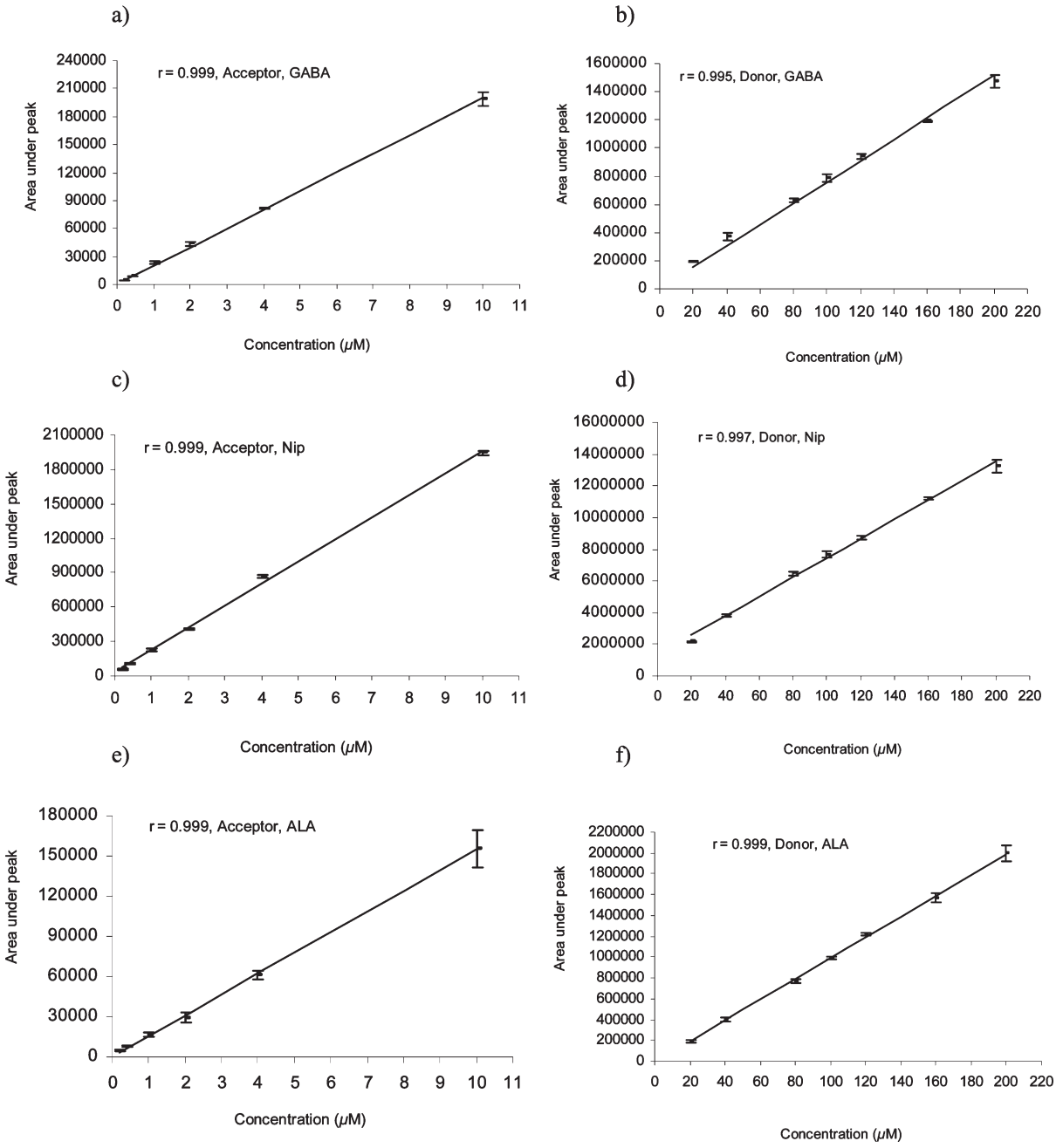

Figure 2. Calibration curves for (a) acceptor samples of GABA, (b) donor samples of GABA, (c) acceptor samples of Nip, (d) donor samples of Nip, (e) acceptor samples of ALA, and (f) donor samples of ALA.

4-Aminobutanoic Acid (GABA). GABA, a noncoded amino acid, is the major inhibitory neurotransmitter in the CNS. Lower amounts of GABA have been observed in several CNS disorders such as epilepsy, Huntington's disease, and Parkinsonism. Increased levels of GABA in the CNS have been proposed for the treatment of these diseases. However, GABA crosses the BBB in an energy- and temperature-dependent manner very poorly and is pumped out from the brain at a greater rate (16 times more) than its BBB influx. Therefore high doses of GABA are required to increase its uptake; however, such doses are associated with severe side effects.[35−38] To overcome this problem, several GABA prodrugs, such as GABA-esters,[39,40] GABA-amide,[41] and GABA-lactame,[35] have been applied and show improved brain uptake.

To improve GABA permeability by passive diffusion, it was coupled to our three peptides. Peptides were evaluated by PAMPA and IAMC (Table 1). The peptides showed good permeability when carrying GABA through the PAMPA membrane and were classified as compounds with medium permeability,[34] whereas PAMPA was not permeable for GABA alone. Anderson et al.[42] reported that GABA benzyl ester with dihydropyridine caused anxiolytic activity in rats, and its oxidized form, pyridinium salt, remained in the brain for 12 h, while GABA alone was ineffective in this case. Carelli et al.[36] also showed that dimer of GABA benzyl ester with dihydropyridine increased the hypnosis time, a typical GABA-mimetic effect, in rats compared with GABA alone. They concluded that the biological activities above are because of the pyridinium salt. Furthermore, Galzigna et al.[41] demonstrated

Table 1. Percentage of Transport, Effective Permeability (P_e), Membrane Retention (%R) after 4 h in the PAMPA and k_{IAM} of GABA, Nip, ALA, L-Dopa, X-BBB-shuttles (X = GABA, Nip, ALA, L-Dopa), and Control Compounds (Propranolol, Carbamazepine)[a]

compd	P_e ($\times 10^6$) cm/s[d]	transport (%) (4 h) [d]	membrane retention (%R) [d]	k_{IAM}[d]
propranolol	7.7	13.9	17.2	1.9
carbamazepine	11.5	19.3	< 1	2.1
GABA	0	0	5.4 ± 1.4	
GABA-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$	0.4 ± 0.06	0.8 ± 0.1	< 1	12.5 ± 2.2
GABA-Cha-(*N*-MePhe)$_3$-CONH$_2$	0.4 ± 0.1	0.8 ± 0.3	16.1 ± 8.3	28.2 ± 6.7
GABA-2Nal-(*N*-MePhe)$_3$-CONH$_2$	0.2 ± 0.02	0.4 ± 0.04	18.4 ± 1.4	30.2 ± 4.4
Nip	0	0	52.6 ± 1.4	
Nip-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$	1.4 ± 0.1	2.8 ± 0.2	4.6 ± 0.4	8.1 ± 1.3
Nip-Cha-(*N*-MePhe)$_3$-CONH$_2$	1.2 ± 0.1	2.5 ± 0.2	19.4 ± 0.7	> 128[b]
Nip-2Nal-(*N*-MePhe)$_3$-CONH$_2$	1.1 ± 0.06	2.4 ± 0.1	23.6 ± 1	30.4 ± 1.5
ALA	0	0	23 ± 0.4	
ALA-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$	1 ± 0.04	2.1 ± 0.2	14.5 ± 1.5	23.1 ± 1.6
ALA-Cha-(*N*-MePhe)$_3$-CONH$_2$	0.3 ± 0.02	0.7 ± 0.03	62 ± 4.9	> 128[b]
L-dopa[c]	0	0	4.3 ± 0.4	0.3
L-dopa-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$[c]	1.1 ± 0.1	2.4 ± 0.2	14.1 ± 2	29.6 ± 1.3
L-dopa-Cha-(*N*-MePhe)$_3$-CONH$_2$[c]	0.7 ± 0.06	1.4 ± 0.1	52.1 ± 1.2	> 128[b]
L-dopa-2Nal-(*N*-MePhe)$_3$-CONH$_2$[c]	0.3 ± 0.1	0.7 ± 0.2	39.3 ± 9	102 ± 6.2

[a] Data are expressed as the mean ± SD. [b] Retention time higher than 60 min in the IAMC HPLC column. [c] These results are from our previous study and are included to facilitate comparison.[14] [d] Parameters definitions and their calculations are provided in Experimental Section.

that *N*-benzoyl-GABA and *N*-pivaloyl-GABA, in contrast to GABA, crossed the BBB in rats and were cleaved enzymatically to release GABA. On the basis of these observations, we hypothesize that GABA-peptides while inside the brain either may be used as substrates for GABA receptors or be degraded proteolytically and release GABA, which because of its incapacity to cross the BBB passively would be trapped the brain and therefore show a longer retention in this organ. Eytan et al.[43] reported that elimination of a drug by P-gp can be overcome by fast transmemberane movement rate. Hence, by greatly improving the permeability of GABA, our peptides may also reduce GABA efflux from the brain.

At this point, we had established that the peptides carry L-dopa and GABA through the PAMPA phospholipid barrier. We then decided to go further and assess the peptides as shuttles for two more distinctive small drugs, Nip and ALA, so as to examine the peptides as versatile BBB-shuttles.

Nipecotic Acid (Nip). Other strategies to increase the extracellular concentration of GABA in the brain and then prevent GABA deficiency include inhibiting its reuptake by neurons and glial cells and direct agonist of GABA receptors. Nip is one of the most potent GABA-reuptake inhibitors and also a GABA receptor agonist.[37,44] However, it does not penetrate the BBB.[45] In mice, Bonina et al.[46] and Manfredini et al.[47] studied the anticonvulsant effect of Nip esters from conjugation with some compounds that enter the brain actively, such as glucose, galactose, and tyrosine[46] and ascorbic acid.[47] Nipecotic esters of glucose and galactose did not show anticonvulsant activity, and the authors concluded that either conjugation caused carbohydrates to lose their affinity for their BBB transporters or carbohydrates were rapidly eliminated by the liver after injection. Conversely, nipecotic esters of tyrosine and ascorbic acid were transported across the BBB; however, these authors could not corroborate whether the observed anticonvulsant activity was because of Nip was released while inside the brain or because of the intact esters. Furthermore, Wang et al.[48] studied the brain delivery of Nip and its *n*-butyl ester after nasal and intravenous administration to rats. While Nip was not detected in the brain, its *n*-butyl ester delivered Nip to the brain. Andersen at el.[49] also found that some *N*-substituted Nip derivatives had an anticonvulsant effect in mice that was greater than that caused by diazepam, an anticonvulsant drug available on the market. The authors observed that when nitrogen of Nip is substituted by an amide, the corresponding analogue loses its activity. On the basis of this observation, they reasoned that presence of the nitrogen of Nip as amine group is crucial for its activity. With these findings in mind, we coupled Nip to the peptides through its carboxyl group to keep the feature of Nip that is required for its activity once inside the brain. We then assessed the capacity of the resulting Nip-peptides to carry Nip across the PAMPA barrier. While Nip alone did not have the capacity to cross the PAMPA phospholipid membrane, all the peptides performed well at shuttling Nip in PAMPA and were categorized as high permeating compounds.[34] The Nip-peptides displayed better permeability than the GABA-peptide constructs. This improvement may be due to the effect of the cyclic conformation of Nip.

5-Aminolevulinic Acid (ALA). ALA, precursor of photosensitizer protoporphyrin IX (PpIX), has been applied for the diagnosis and photodynamic therapy (PDT) of cancer, including esophageal, bladder, lung, breast, and brain cancer. In cancer therapy, PDT is an approach in which malignant cells, which show higher expression of PpIX than healthy cells after administration of ALA or ALA prodrugs, are destroyed following irradiation with light.[50−52] Although several transport systems have been found for ALA in a range of tissues and cell lines, however these transporters were not observed at the BBB.[53−56] Ennis et al.[55] found that ALA passed the BBB poorly by passive diffusion, while Garcia et al.[57] and Terr et al.[58] observed that ALA did not enter the brain and isolated brain capillaries.

To enhance the efficiency of PDT, ALA should penetrate malignant cells sufficiently; however, the movement of ALA through cell membranes is limited due to its hydrophilic character. To enhance ALA permeation, it has been used in several forms such as esters,[59] dendrimers,[60] and peptides[61−63] and has been entrapped in liposome.[64] ALA hexyl ester enters the brain 7-fold more than free ALA; however, it is toxic in the absence of light and in tumor cultures it decreases PpIX formation.[59,64]

The use of peptides to deliver ALA to cells is a relatively recent approach which shows great potential. Of several derivatives studied, Berger et al.[61,62] demonstrated that the amino acid derivative of ALA with phenylalanine, attached through the amino group of ALA, was the best precursor for PpIX formation in cells. They then prepared dipeptide-ALA derivatives that were also converted to PpIX with less toxicity in the absence of light compared to the amino acid-ALA derivatives. Later Bourre et al.[63] found that ALA derivative with acetylated Phe is also 5 times more efficacious than ALA in producing PpIX in cells. To evaluate the capacity of our peptides to enhance ALA permeation through membranes, ALA-peptides were synthesized on solid-phase and studied by PAMPA and IAMC. Compound ALA-2Nal-(*N*-MePhe)$_3$-CONH$_2$ was excluded from this study because it was not obtained with sufficient purity to be used for the assay. Using PAMPA as an in vitro model of the BBB, two other peptides transferred ALA across the PAMPA membrane while ALA alone did not show permeability, which is consistent with an earlier study by Garcia et al.[57] reporting that ALA did not cross the BBB in rats. ALA-Cha-(*N*-MePhe)$_3$-CONH$_2$ exhibited greater lipophilicity and was expected to be more permeating than ALA-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$; however, this was not the case, probably because this compound is too lipophilic and it caused the peptide to attach to the PAMPA membrane.

For all the selected cargos, to show that the compounds in the acceptor wells of the PAMPA plate were intact cargo-peptides and not fragments formed during the assay, all samples of acceptor wells were examined by both HPLC and MALDI-TOF MS. Our peptides have been able to shuttle these cargoes across PAMPA barrier. It has been reported in the literature that esterification of the selected cargoes increased their permeability.[39,40,48,59] Taking this into account, it could not be excluded that the elimination of the cargoes negative charge may, in part, play a role in their permeability. In addition, toxicity is a key issue in this field. From this point of view, the lack of toxicity of our shuttles[14] can be advantageous when compared to other strategies.[40,59]

Permeability, Membrane Retention, Lipophilicity, and Hydrogen Binding. IAMC, in which a phospholipid is covalently bonded to silica particle support, models the partitioning of compounds into biological membranes. The retention time of a compound on an IAMC column is converted to the capacity factor (k_{IAM}) following eq 3 (see Experimental Section), which is applied as a predictor of membrane partition coefficient. k_{IAM} was determined for eight cargo-peptide constructs (Table 1). Most of the cargo-peptide products showed lower capacity factors in IAMC compared to their parent peptides, which exhibited capacity factors higher than 128.[14] This observation may be explained by an increase in H-bond number. For all cargos, the peptide with *N*-MePhe as *N*-terminal amino acid formed fewer H-bonds with solvent water, and therefore it was expected to show a greater capacity factor or lipophilicity in IAMC. However, in contrast, this peptide demonstrated the smallest capacity factor (12.5, 8.1, and 23.1 with GABA, Nip and ALA, respectively, Table 1). This difference may be considered the effect of the side chains of *N*-terminal amino acids: phenyl, cyclohexyl, and naphthyl. These findings show that Cha confers greater lipophilicity to the peptide than 2Nal and *N*-MePhe, while 2Nal increases peptide lipophilicity more than *N*-MePhe. In addition, smaller amounts of the cargo-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ constructs remained in the PAMPA membrane compared with the other cargo-peptides (1 <, 4.6, and 14.5 with GABA, Nip and ALA, respectively, Table 1). These results are consistent with those obtained by IAMC. For our cargo-peptide constructs, there was a good correlation (r = 0.808) between log k_{IAM} and log %R, thereby indicating that lipophilicity (k_{IAM}) is one of the factors that governs the affinity of compounds to remain in the PAMPA membrane. This correlation also shows that the interaction of solute with IAMC phospholipid resembles that of PAMPA.

The logarithms of peptide permeability (log p_e) did not correlate with the logarithms of their lipophilicity from IAMC (log k_{IAM}). These results are in disagreement with those reported by Adejare et al.[65] and Salminen et al.[66] who found acceptable relationships (r = 0.767 and 0.576, respectively) between the blood−brain partition coefficient (log BB) and log k_{IAM} for a set of 21 and 26 structurally diverse compounds, respectively. However, our results are in line with those reported by Chikhales et al.,[67] who showed poor correlation between lipophilicity from octanol−buffer partition coefficient and permeability of *N*-MePhe-content peptides across BBMECs (r = 0.389) or permeability of in situ rat brain perfusion (r = 0.155). Chikhales et al.[67] and Conradi et al.[68] demonstrated the negative effect of H-bond number on peptide permeation in BBMECs, in situ BBB, and Caco-2 cells. In contrast, we found no correlation between H-bond number and permeability of our peptides in PAMPA. This could be attributed to the fact that the inverse relationship between number of H-bonds and permeability is correct only for a structurally related series of compounds such as a family of steroid hormones[69] or peptides differ only between number of *N*-methylated amide bonds.[67,68] However, the number of H-bonds correlated with log k_{IAM} (r = 0.607) and log %R (r = 0.528), thereby indicating that number of H-bonds can predict membrane retention in PAMPA and the capacity factor of IAMC better than PAMPA permeability for this series of compounds. Molecular weight, number of H-bonds, lipophilicity, and properties of amino acids, among others, are factors that influence the capacity of a given peptide to cross membrane phospholipids. On the basis of these results, we conclude that H-bond number or lipophilicity alone is not sufficient to interpret the penetration of peptides into the PAMPA membrane.

Peptide Permeability across the Endothelial Cell Monolayer. The BBMEC model is one of the cell-based BBB models that has been used extensively to screen drug permeation to the brain[67,70−73] and study the capacity of compounds to carry drugs across an in vitro BBB model.[74] This model shows many BBB properties, including tight junction formation, and P-glycoprotein (P-gp) expression.[70] The permeability of Nip-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, Nip-Cha-(*N*-MePhe)$_3$-CONH$_2$, Nip-2Nal-(*N*-MePhe)$_3$-CONH$_2$, and ALA-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, peptides that showed the highest permeability in PAMPA, and Ac-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ as the parent peptide,[14] was examined in BBMECs (Table 2) and results were compared with those from PAMPA to assess the correlation of these two assays. Propranolol, carbamazepine, and caffeine were also tested as compounds that cross the in vivo BBB by passive diffusion. All the peptides and controls showed higher permeability values in BBMECs than in PAMPA. This finding could be attributed to PAMPA being stricter than BBMECs as a result of a greater membrane thickness in PAMPA than the in vivo BBB (125000 nm vs

Table 2. Percentage of Transport, Apparent Permeability (P_{app}), Membrane Retention (%R) after 2 h in BBMEC model of X-*N*-MePhe-(*N*-MePhe)$_3$-$CONH_2$ (X = Ac and ALA), and Nip-Y-(*N*-MePhe)$_3$-$CONH_2$ (Y = *N*-MePhe, Cha 2Nal) and Control Compounds (Propranolol, Carbamazepine, and Caffeine)[a]

compd	P_{app} ($\times 10^6$) cm/s	transport (%) (2 h)	membrane retention (%R)
propranolol	16.9 ± 2.3	21.2 ± 2.7	63.0 ± 3.1
carbamazepine	16.5 ± 1.8	20.0 ± 2.3	67.5 ± 2.5
caffeine	16.1 ± 2	20.6 ± 2.2	67.4 ± 2.2
Ac-*N*-MePhe-(*N*-MePhe)$_3$-$CONH_2$	8.3 ± 0.7	9.8 ± 0.8	80.2 ± 1.6
Nip-*N*-MePhe-(*N*-MePhe)$_3$-$CONH_2$	6.6 ± 0.3	7.9 ± 0.4	74.0 ± 0.2
Nip-Cha-(*N*-MePhe)$_3$-$CONH_2$	5.2 ± 0.6	6.2 ± 0.7	71 ± 2.8
Nip-2Nal-(*N*-MePhe)$_3$-$CONH_2$	3.5 ± 0.04	4.1 ± 0.04	83.8 ± 1.2
ALA-*N*-MePhe-(*N*-MePhe)$_3$-$CONH_2$	15 ± 0.04	17.8 ± 0.05	50.7 ± 0.8

[a] Data are expressed as the mean ± SD.

200−500 nm).[24] ALA-*N*-MePhe-(*N*-MePhe)$_3$-$CONH_2$ presented the greatest permeation and was comparable to that of positive controls. The permeability values for the three peptides with Nip in BBMECs showed excellent correlation (r = 0.970) with PAMPA values. Given that the BBMEC model is predictive of BBB permeability in vivo[71] and PAMPA showed excellent correlation with BBMECs, PAMPA, which is simpler and cheaper than the latter, can be consider an in vitro BBB model for predicting BBB permeation by passive diffusion.

Peptide Families Containing Proline. We synthesized a five-member peptide library with proline as the amino acid on solid-phase [Ac-(Pro)$_n$-$CONH_2$ (n = 2, 4, 6, 8, 10)] to study the permeability and lipophilicity of these compounds in PAMPA and IAMC, respectively. We also compared them with those from *N*-MePhe-rich peptides in order to establish whether the in vitro BBB transport properties of our versatile BBB shuttles are due to the presence of secondary amides. While peptides using *N*-MePhe as amino acid showed great permeability in PAMPA,[14] the replacement of *N*-MePhe by proline dramatically decreased peptide permeation and lipophilicity (Table 3). This result is attributed to proline being a less hydrophobic amino acid compared to *N*-MePhe and as such caused the peptides to be excessively hydrophilic to interact with the phospholipids of PAMPA and IAMC. Thus the peptides with proline did not show permeability and membrane retention (%R) in PAMPA and exhibited smaller retention times in IAMC than that from citric acid (reference compound that is not retained in the IAMC column). We postulate that the great difference between the permeability and lipophilicity of proline family and the *N*-MePhe family is the result of the phenyl group of *N*-MePhe.

Table 3. Percentage of Transport, Effective Permeability (P_e) Membrane Retention (%R) after 4 h in the PAMPA and k_{IAM} of Ac-(Pro)$_n$-$CONH_2$ (n = 2, 4, 6, 8, 10) and Control Compounds (Propranolol, Carbamazepine)

compd	P_e ($\times 10^6$) cm/s	transport (%) (4 h)	membrane retention (%R)	k_{IAM}
propranolol	7.9	14.1	22	1.9
carbamazepine	8.1	14.4	< 1	2.1
Ac-(Pro)$_2$-$CONH_2$	0	0	0	*a*
Ac-(Pro)$_4$-$CONH_2$	0	0	0	*a*
Ac-(Pro)$_6$-$CONH_2$	0	0	0	*a*
Ac-(Pro)$_8$-$CONH_2$	0	0	0	*a*
Ac-(Pro)$_{10}$-$CONH_2$	0	0	0	*a*

[a] Retention time in the IAMC HPLC column smaller than that of reference compound (citric acid).

Conclusions

Here, we evaluated the versatility of three tetrapeptides to transport distinct drugs through an artificial membrane and endothelial cells by passive diffusion. For this purpose, the peptides were attached to GABA, Nip, and ALA and their permeability and lipophilicity were assayed in PAMPA and IAMC. The peptides allowed transport of the cargos and notably achieved success as BBB shuttles for these drugs, which alone did not show permeability in PAMPA. While the peptides showed high capacity to transfer Nip across the PAMPA membrane, they showed less capacity for GABA. However, for all the cargos studied, the peptide with *N*-MePhe as the *N*-terminal amino acid showed the best permeability. Some of the peptides also were tested in BBMECs and showed higher permeability values than in PAMPA. Given these results, these three peptides could provide the means to deliver drugs, which cannot enter the brain unaided, into the CNS by passive-diffusion transport. The use of the BBB shuttles could pave the way for improving brain drug delivery of small neurodrugs that lack the capacity to reach their site of action in the CNS.

Experimental Section

Materials and Methods. Protected amino acids and resins were supplied by Luxembourg Industries (Tel-Aviv, Israel), Neosystem (Strasbourg, France), Calbiochem-Novabiochem AG (Laüfelfingen, Switzerland), Bachem AG (Bubendorf, Switzerland), or Iris Biotech (Marktredwitz, Germany). PyBOP was supplied by Calbiochem-Novabiochem AG. DIEA, ninhydrin, and *ss*-mercaptoethanol were obtained from Fluka Chemika (Buchs, Switzerland). HOAt was purchased from GL Biochem Shanghai Ltd. (Shanghai, China). Solvents for peptide synthesis and RP-HPLC were obtained from Scharlau or SDS (Barcelona, Spain). Trifluoroacetic acid was purchased from KaliChemie (Bad Wimpfen, Germany). Collagen type IV, Fibronectine, 8-(4-chlorophenylthio(CPT))-cAMP, $NaHCO_3$, MEM nonessential amino acids, and HEPES were purchased from Sigma Aldrich (USA), RO-20-1724 from Calbiochem (USA), DMEM from Biological Industries (Israel), and fetal bovine serum from PAA Laboratories GmbH (Austria). Other chemicals used were purchased from Aldrich (Milwaukee, WI) and were of the highest purity commercially available. PAMPA plates and PAMPA system solution were from pION (Woburn, MA). Porcine polar brain lipid extract (PBLEP) was purchased from Avantis Polar Lipids (Alabaster, AL). IAMC column (10 mm × 4.6 mm, 12 μm, 300 Å, IAM.PC.DD2 column, Regis Technologies Inc., Morton Grove, IL). Mass spectra were recorded on a MALDI Voyager DE RP time-of-flight (TOF) spectrometer (PE Biosystems, Foster City, CA), using an ACH matrix. High resolution mass spectra were recorded on a LTQ-FT Ultra (Thermo Scientific). HPLC chromatograms were

recorded on a Waters model Alliance 2695 with Photodiode array detector 996 from Waters (Waters, Milford, MA) using a Symmetry C_{18} column (150 mm × 4.6 mm × 5 μm, 100 Å, Waters); solvents: H_2O (0.045% TFA) and MeCN (0.036% TFA); flow: 1 mL/min and software Millenium version 4.0. HPLC-MS [Waters model Alliance 2796, quaternary pump, UV/vis dual absorbance detector Waters 2487, ESI-MS model Micromass ZQ and Masslynx version 4.0 software (Waters)] was done using a Symmetry 300 C_{18} column (150 mm × 3.9 mm × 5 μm, 300 Å, Waters), solvents: H_2O (0.1% formic acid) and MeCN (0.07% formic acid), flow: 1 mL/min. The products were purified in a Waters 600 with dual absorbance detector (Waters 2487 Waters), and a Symmetry C_{18} column (100 mm × 30 mm × 5 μm, 100 Å, Waters), solvents H_2O (0.1% TFA) and MeCN (0.05% TFA), flow: 10 mL/min. Purity was checked by reverse-phase HPLC using a Symmetry C_{18} column. Products showed purity ≥95.

General Protocols for Solid-Phase Synthesis. Syntheses were performed on a 100 μmol-scale/each, in all cases L-amino acids were used. Solid-phase peptide elongation and other solid-phase manipulations were done manually in polypropylene syringes, each fitted with a polyethylene porous disk. Solvents and soluble reagents were removed by suction. Washings between synthetic steps were done with DMF (5 × 30 s) and DCM (5 × 30 s) using 5 mL of solvent/g resin each time. During couplings, the mixture was allowed to react with intermittent manual stirring.

Identification Tests. The Kaiser colorimetric assay[75] was used for the detection of solid-phase bound primary amines, while the De Clercq test[76] was used for secondary amines bound to solid-phase.

Initial Conditioning of Resin. The Sieber resin[77] was conditioned by washing with DCM (5 × 30 s) and DMF (5 × 30 s) followed by a 20% piperidine solution in DMF (2 × 1 min and 1 × 10 min) to remove the Fmoc group. Finally, the resin was washed with DMF (5 × 30 s).

Fmoc Group Removal. The Fmoc group was removed by treating the resin with 20% piperidine in DMF (3−4 mL/g resin, 2 × 1 min and 1 × 10 min). To remove the Fmoc group from Fmoc-*N*-MePhe-OH and Fmoc-Pro-OH, an additional treatment with DBU, toluene, piperidine, and DMF (5%:5%:20%:70%) (1 × 5 min) was performed.

Coupling Methods. Method 1, Coupling of the First Amino Acid onto the Sieber Resin. *N*-Protected *N*-methylated phenylalanine (4 equiv, 160.5 mg), PyBOP (4 equiv, 208 mg), and HOAt (12 equiv, 163.3 mg) were added sequentially to the resin in DMF (3 mL) followed by DIEA (12 equiv, 204 μL). The mixture was allowed to react with intermittent manual stirring for 1.5 h. The solvent was removed by filtration; the resin was washed with DMF (5 × 30 s) and DCM (5 × 30 s). The extent of coupling was checked by the Kaiser colorimetric assay.

Method 2, Coupling of Second Amino Acid and the Following Amino Acid onto the Sieber Resin. The procedure was the same as for the first one except that *N*-protected phenylalanine was used. The coupling was repeated two more times and the extent of coupling was checked by the De Clercq test.

Method 3, Synthesis of Peptides Containing Proline. The procedure was the same as the method 2, except that *N*-protected proline was used.

Amino acid *N*-alkylation. The *N*-methylation of phenylalanine was performed using the method described by Miller.[78] This process can be divided into three steps: (A) protection and activation with *o*-nitrobenzenesulfonyl chloride (*o*-NBS), (B) deprotonation and methylation, and (C) *o*-NBS removal.

A. Protection and Activation with *o*-NBS. To perform the protection, *o*-NBS (3 equiv, 67 mg) and collidine (5 equiv, 66 μL) in DCM were added to the resin. The reaction was left with intermittent manual stirring for 1 h, and this step was repeated once and checked by the Kaiser test.

B. Deprotonation and Methylation. Methyl *p*-nitrobenzensulfonate (4 equiv, 86.9 mg) and MTBD (3 equiv, 43 μL) in DMF were added to the resin and left for 30 min, and this step was repeated once.

C. *o*-NBS Removal. To remove *o*-NBS, *ss*-mercaptoethanol (10 equiv, 70 μL) and DBU (5 equiv, 75 μL) in DMF were added to the resin and the mixture was left to react for 10 min under a nitrogen atmosphere. This process was repeated once for 40 min.

Nip Coupling. Fmoc-Nip-OH (4 equiv, 140.5 mg), PyBOP (4 equiv, 208 mg), and HOAt (12 equiv, 163.3 mg) were sequentially added to the resin in DMF (3 mL) followed by DIEA (12 equiv, 204 μL). The mixture was allowed to react with intermittent manual stirring for 1.5 h. The solvent was removed by filtration, and the resin was washed with DMF (5 × 30 s) and DCM (5 × 30 s). The coupling was repeated two more times in the case of coupling on *N*-MePhe. The extent of coupling was checked by the De Clercq test or the Kaiser colorimetric assay.

GABA/ALA Coupling. The procedure was the same as for Nip, except that Fmoc-GABA-OH or Fmoc-ALA-OH was used.

Cleavage of the Peptides. Final amide peptides were cleaved from the resin using 2% TFA in DCM (6 × 3 min).

Product Workup and RP-HPLC Purification. After cleavage of the peptides, the solvent was evaporated by N_2. The residue was dissolved in H_2O:MeCN (1:1) and then lyophilized. The peptides were purified by reverse-phase HPLC using a symmetry C_{18} column (100 mm × 30 mm × 5 μm, 100 Å, Waters), at a 10 mL/min flow rate with the following solvents: A, H_2O with 0.1% TFA; B, MeCN with 0.05% TFA.

Product Characterization. The identity of the compounds synthesized was confirmed using MALDI-TOF MS, HPLC-MS, and HRMS. Purity was checked by reverse-phase HPLC using a symmetry C_{18} column. Peptides showed purity ≥95 (see Supporting Information).

Parallel Artificial Membrane Permeability Assay (PAMPA). The PAMPA[15] was used to determine the capacity of compounds to cross the BBB by passive diffusion. The effective permeability of the compounds was measured at an initial concentration of 200 μM. The buffer solution was prepared from a concentrated one, commercialized by pION, and following the manufacturer's instructions. pH was adjusted to 7.4 using a NaOH 0.5 M solution. The compound of interest was dissolved in buffer solution or water (in the case of cargos alone, evaluated by HPLC-MS) and 1-propanol (20%, cosolvent) to the desired concentration (200 μM). The PAMPA sandwich was separated, and the donor well was filled with 200 μL of the compound solution of interest. The acceptor plate was placed into the donor plate, ensuring that the underside of the membrane was in contact with buffer. Four μL of the mixture of phospholipids (20 mg/mL) in dodecane was added to the filter of each well, and 200 μL of buffer solution was added to the each acceptor well. The plate was covered and incubated at room temperature in a saturated humidity atmosphere for 4 h under orbital agitation at 100 rpm. After the 4 h, 150 μL/well from the donor plate and 150 μL/well from the acceptor plate were transferred to HPLC vials and 100 μL/each sample were injected into a HPLC reverse-phase Symmetry C_{18} column (150 mm × 4.6 mm × 5 μm, 100 Å, Waters). Transport was also confirmed by MALDI-TOF spectrometry in order to check that the compound had kept its integrity. For PAMPA assays evaluated by HPLC-MS, the same procedure was used except that buffer solution was replaced by water and after the 4 h, 100 μL/well from the donor plate and 100 μL/well from the acceptor plate were transferred to HPLC-MS vials and 10 μL/each donor and 20 μL/each acceptor were injected into a HPLC-MS apparatus.

The phospholipid mixture used was a porcine polar brain lipid extract. Composition: phosphatidylcholine (PC) 12.6%, phosphatidylethanolamine (PE) 33.1%, phosphatidylserine (PS) 18.5%, phosphatidylinositol (PI) 4.1%, phosphatidic acid 0.8%, and 30.9% of other compounds. The effective

permeability after 4 h was calculated using eq 1 and also the percentage of transport after was calculated using eq 2.

$$P_e = \frac{-218.3}{t} \times \log\left[1 - \frac{2C_A(t)}{C_D(t_0)}\right] \times 10^{-6}\text{cm/s} \quad (1)$$

$$T\% = \frac{C_A(t)}{C_D(t_0)} \times 100 \quad (2)$$

where t is time (h); $C_A(t)$ is the compound concentration in the acceptor well at time t and $C_D(t_0)$ is the compound concentration in the donor well at 0 h. The membrane retention (%R) was calculated from the difference between the total starting amount and the amounts in donor and acceptor compartments at the end of the experiment (t = 4 h).

Immobilized Artificial Membrane Chromatography (IAMC). Retention times were determined using an IAMC column with phosphatidylcholine (PC), the major phospholipid in cell membranes, which was covalently immobilized (10 × 4.6 mm, 12 μm, 300 Å, IAM.PC.DD2 column, Regis Technologies Inc.).

The compounds were detected by UV absorption at 220 nm. The chromatograms were obtained using an HPLC working isocratically with a mobile phase containing 10 mM phosphate buffer, 2.7 mM KCl and 137 mM NaCl at pH 7.4 and 20% (v/v) MeCN. The retention times (t_R) were transformed into capacity factors (k_{IAM}) following eq 3,

$$k_{IAM} = (t_R - t_0)/t_0 \quad (3)$$

where t_R is the compound retention time (min), and t_0 is the citric acid retention time (min) indicating the column dead time.

HPLC-MS Experiment for Quantification. The HPLC-MS analysis was performed using a Waters 2795 separation module, operating in positive ion mode with a Waters 2487 dual λ absorbance detector. The HPLC column used was a reverse phase symmetry column (4.6 mm × 150 mm, 5 μm, C_{18}). The samples were eluted at 1 mL/min using different linear gradients of solvents A (H_2O containing 0.1% formic acid (v/v)) and B (MeCN containing 0.07% formic acid (v/v)). The detection was performed at 220 nm. External standards at concentration of 0.2, 0.4, 1, 2, 4, 10, 20, 40, 80, 100, 120, 160, and 200 μM were prepared by diluting the 1000 μM stock solution with 20% 1-propanol in water. Prior to the experiments, the compounds were analyzed by HPLC-MS using scan mode to determine the retention time of each. For the experiments, single-ion monitoring (SIM) was used instead of a scan mode.

Cell Culture. The in vitro model was establish using a coculture of bovine brain microvessel endothelial cells (BBMECs) obtained from Cell Applications (USA) and newborn rat astrocytes, as previously described.[79] In brief, before cell coculture (24-wells Transwell polycarbonate with a surface area of 0.33 cm^2 and pore-size of 0.4 μm, Corning Costar), the upper surface of plate inserts was coated with collagen type IV and fibronectin. Next, the inserts were placed upside down in a large Petri dish and 40 μL of a suspension (containing approximately 45000 astrocytes) was placed on the bottom of each filter. The Petri dish was put in an incubator for 1 h and 40 μL of fresh DMEM+S was added to the bottom of each filter every 15 min. The inserts were then transferred back into the plate and incubated at 37 °C, 10% CO_2 for three days. After three days, 2 h before seeding the BBMECs, the medium was replaced by DMEM+S supplemented with 125 μg/mL of heparin. Two hours later, BBMECs were seeded in the inserts (45000 cells per filter). The plate was kept in an incubator at 37 °C, 10% CO_2 for three more days. After three days of coculture, the medium was replaced by DMEM+S supplemented with cAMP and RO-20-1724, and kept at 37 °C and 10% CO_2. On day 8 of coculture, transendothelial electrical resistance (TEER) measures showed that the system was ready for transport studies.

Transport Studies. Inserts were washed with Ringer/HEPES solution (150 mM NaCl, 5.2 mM KCl, 2.2 mM $CaCl_2$, 0.2 mM $MgCl_2$, 6 mM $NaHCO_3$, 2.8 mM glucose, 5 mM HEPES). Acceptor compartments were filled with the Ringer/HEPES solution and donor compartments with the peptides at a concentration of 100 μM in Ringer/HEPES solution with a final concentration of 1% DMSO. The plate was incubated for 2 h at 37 °C and 10% CO_2. After the experiments, donor and acceptor compartments were analyzed by HPLC. All the peptides were tested in triplicate.

Permeability calculations were performed following eq 4[74,80,81]

$$P_{app} = (dQ/dt)(1/A)(1/C_0)(\text{cm/s}) \quad (4)$$

Where (dQ/dt) is the amount of compound present in the acceptor compartment in function of time (nmol/s), A is the area of the insert (cm^2), and C_0 is the initial concentration of peptide placed in the donor compartment (nmol/mL).

TEER Measurement. The TEER was determined by using an ohmmeter Millicell ERS system (MERS 000 01, Millipore). TEER measures confirmed the formation of a functionally intact in vitro BBB by day 8 of coculture. The TEER values represent the tightness or integrity of the in vitro BBB. The TEER value (mean ± SD) for all wells was 141 ± 5.7 Ω/cm^2.

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Supporting Information Available: Characterization of all peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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