Toward an Optimal Blood−Brain Barrier Shuttle by Synthesis and Evaluation of Peptide Libraries

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Several peptide families containing N-methylated amino acids were designed and synthesized using solid-phase peptide synthesis (SPPS). The permeability and phospholipophilicity of these compounds were studied by parallel artificial membrane permeability assay (PAMPA) and immobilized artificial membrane chromatography (IAMC) to select the best peptides in terms of length, terminal groups, and amino acid replacement to be used as carriers that pass through a model of the blood−brain barrier (BBB) by passive diffusion. Furthermore, the enzymatic stability of these peptides in human serum and their cell viability by MTT assay were tested. These peptide families showed great stability and nontoxicity. The three peptides that showed the greatest permeability were coupled to levodopa (a nonpassive permeating drug) and assessed. These peptides effectively transferred levodopa through an artificial membrane by means of passive diffusion.

Introduction

The blood−brain barrier (BBB[a]) is a membrane that protects the brain from harmful substances in circulating blood and regulates the entry of particular molecules from blood into the central nervous system (CNS). This physical and enzymatic barrier is the major bottleneck for the delivery of therapeutic agents to the brain. The BBB is formed by endothelial cells that are sealed through tight junctions, which significantly block paracellular transport.[1–5]

To bypass the BBB and deliver drugs to the brain, several strategies have been used: temporarily opening the BBB, administration of very high doses of a drug, and direct injection of a drug into the spinal cord. However, these approaches imply risks of infection and toxicity and, in addition, require qualified personnel.

Other strategies have been developed such as modifying the structure of a drug to increase its permeability by passive diffusion. Another option is to couple a drug to a "Trojan horse", a compound that passes the BBB by receptor-mediated transcytosis (RMT) and can carry the drug across the barrier.[1,5–10]

The concept of Trojan horses can be extended to other mechanisms, in addition to RMT, by means of searching for peptides with the capacity to enter the brain by passive diffusion and shuttle drugs that cannot cross the BBB unaided. These diffusion "Trojan horses", also called BBB-shuttles, have recently been described by our group.[11]

The main physicochemical properties that determine whether a compound can cross the BBB are lipophilicity and molecular weight (MW), among others. Lipophilicity can be affected by the presence of polar groups or H-bond donors/acceptors in the structure of a compound. Compounds with a MW above 400−600 Da show poor capacity to cross the BBB.[9,12–16] However, the effect of MW remains unclear[2,17] because there are a number of BBB-penetrating compounds (BBB+) of more than 500 Da[18] and lipid solubility can neutralize the negative effect of MW. Although there is a good inverse correlation between BBB permeability and MW for lipid-insoluble compounds, this correlation is very poor for lipid-soluble ones ($r = 0.105$). In contrast, for a series of structurally related compounds (for example, *n*-alcohols), molecules with higher MWs show higher lipid solubility and are expected to have greater BBB permeability.[19]

The above physicochemical properties, together with peptide length and amino acid sequence, are determinants of the capacity of peptides to cross the BBB.[2,20] Among these properties, the number of potential hydrogen bonds that the compound can form is a key element.[21,22]

N-Methylation of amino acids has been proposed as a powerful tool to decrease the number of H-bonds and increase the lipophilicity and permeability of peptides. *N*-Methylamino acids are not rare in nature. *N*-Methylphenylalanine (*N*-MePhe) is present in several natural peptides and proteins, such as haliclamide from the marine sponge *Haliclona* sp., which shows in vitro antitumor activity,[23] Pili protein from *Pseudomonas aeruginosa,* which mediates the infectious action of a number of *Pseudomonas* bacteriophages,[24–26] and staphylomycins, a peptide antibiotic produced by *Streptomyces virginiae*.[27]

Given these properties and the observation that *N*-MePhe is present in several natural peptides, *N*-MePhe was chosen as a key residue for our design of shuttles that cross the BBB by passive diffusion.

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[a] Abbreviations: ACH, α-cyano-4-hydroxycinnamic acid; Ac_2O, acetic anhydride; BBB, blood−brain barrier; CNS, central nervous system; Da, dalton; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; 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; MMT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 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-yloxytripyrrolidinophosphonium hexafluorophosphate; SPPS, solid-phase peptide synthesis; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; t_R, retention time.

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For this purpose, several peptide libraries were synthesized and evaluated by PAMPA (parallel artificial membrane permeability assay) and IAMC (immobilized artificial membrane chromatography). Our findings indicate that the presence of *N*-methylamino acids in a peptide sequence improves its lipophilicity and permeability. In addition, these amino acids increase peptide stability against enzymatic cleavage. Also, the use of unnatural hydrophobic amino acids can be a powerful tool to achieve BBB-shuttles with improved permeability properties and proteolytic stability.

Results and Discussion

Library of Peptides Containing *N*-MePhe. To evaluate the effect of length on peptide permeability, a library of nine peptides, Ac-(*N*-MePhe)$_n$-CONH$_2$ ($n = 2-10$), which have only *N*-MePhe in their sequences, was synthesized using a solid-phase peptide synthesis (SPPS) technique with Fmoc strategy, as it has several advantages over the Boc strategy.[28] Although synthesis of highly N-methylated peptides is not an easy task,[29] we attempted to synthesize this library for two reasons. First, it has been reported that the *N*-methylphenylalanine trimer, Ac-(*N*-MePhe)$_3$-CONH$_2$, crosses the BBB in situ,[21] and second, peptide bond between two phenylalanines is one of the major sites for proteolytic degradation.[30] Furthermore, N-methylation of peptide bonds has been widely used to enhance the stability of these compounds in response to enzymatic proteolysis while simultaneously retaining their bioactivity.[31–34]

All phenylalanines were methylated on solid support[35] except the first. Attempts to methylate it failed, so the commercially available *N*-MePhe was used. All peptides were characterized using HPLC, HPLC−MS, MALDI-TOF MS, and HRMS. All peptides showed purity greater than 98%. N-Methylation in solid-phase instead of using N-methylated amino acid is cheaper and reduces DKP formation during coupling and Fmoc deprotection. Performed in solid-phase, N-methylation can also open up a new avenue for the preparation of N-methylated amino acids that are not commercially available. All peptides were acetylated in the N-terminal amino acid position on solid support without losing Ac-*N*-MePhe during the cleavage or needing to be acetylated in solution that sometimes is not achievable. One of the difficulties of poly-N-methylated peptide synthesis is the formation of byproduct during the cleavage of the peptide from the resin. Using Sieber resin, which requires only 2% TFA for cleavage, we did not observe any byproduct formation.[29]

Evaluation of Peptide Permeability by PAMPA. There are several cell-based assays to predict the permeability of compounds across the BBB, such as bovine brain microvessel endothelial cells (BBMEC),[36,37] porcine brain microvessel endothelial cells (PBMEC),[38,39] rat brain microvascular endothelial cells (rBMEC),[40] and Madin Darby canine kidney cells (MDCK).[41] However, all these require cell culture and are time-consuming. Moreover, since these assays involve both passive and active mechanisms, they are not appropriate for measuring only passive diffusion.

The PAMPA, a relatively new technique,[42] is an approach based on an artificial membrane system with a microfilter coated by phospholipids. Depending on the phospholipid type, this assay can be used to predict oral absorption,[43] human skin permeation (PAMPA-skin),[44,45] and BBB permeation (PAMPA-BBB).[46] PAMPA values are in accordance with those from MDCK and in vivo.[47] Given that active transporters are not involved in PAMPA and therefore the permeability measured is exclusively due to passive diffusion, we used this assay to determine the permeability of our peptides. Lecithin in dodecane has been used as a phospholipid,[42] but it does not perfectly discriminate between CNS− and CNS+ compounds. We used a porcine polar brain lipid extract because it better mimics the lipid composition of endothelial cells of the BBB.[46] In vitro models to determine permeability across a membrane include a stagnant water layer called the unstirred water layer (UWL), whose thickness in brain capillaries is nearly zero. This layer can be thinned by stirring the solution in in vitro assays.[48–51] Thus, to better mimic the BBB in vivo, we performed all PAMPAs by placing the PAMPA plate on an orbital shaker at 100 rpm.

Table 1. Effect of 20% 1-Propanol (Cosolvent) on the Effective Permeability (P_e) of Propranolol, Carbamazepine, Caffeine, L-Dopa, and Dopamine in the PAMPA[a]

	system solution		system solution + 20% 1-propanol	
compd	P_e ($\times 10^6$), cm/s	transport (4 h), %	P_e ($\times 10^6$), cm/s	transport (4 h), %
propranolol	8.8 ± 1.3	15.5 ± 1.9	8.4 ± 1.7	14.9 ± 2.6
carbamazepine	9.7 ± 0.7	16.8 ± 1.1	12.8 ± 3.8	20.6 ± 4.8
caffeine	1.8 ± 0.05	3.6 ± 0.1	1.8 ± 0.2	3.6 ± 0.5
L-dopa	0	0	0	0
dopamine	0	0	0	0

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

Some of our peptides were not completely soluble in the commercial system solution used for the PAMPA. To overcome this problem, the solution can be filtered before the assay, but sometimes the amount of compound in the solution after filtration is too low to be detectable, especially for sparingly soluble compounds.[52] Another recommended approach is the addition of a cosolvent to the solution.[48,53,54] PAMPA has a purely artificial membrane, so the percentage of cosolvent can be increased to 30%, while for cell-based assays this percentage is limited (≤1%) because of the toxicity effect of cosolvent.[53] In our study several cosolvents, such as acetonitrile, ethanol, DMSO, and 1-propanol, were examined in a range of percentages (max 20%). When 20% 1-propanol was used, all our peptides were completely soluble; therefore, all PAMPAs were performed with 20% of 1-propanol as a cosolvent. First, we evaluated the effect of 20% 1-propanol on permeability in the PAMPA and phospholipid stability. For this purpose, we chose propranolol, carbamazepine, and caffeine as compounds with the ability to cross the BBB by passive diffusion, and L-dopa and dopamine as compounds that do not enter the brain passively. The permeability of these compounds was examined in the presence and absence of 20% 1-propanol (Table 1). While Sugano et al.[53] reported that DMSO, ethanol, and PEG 400 decreased the permeability of propranolol and Avdeef[48] observed the same effect with 10% 1-propanol in PAMPA assays, in our study 20% 1-propanol did not affect the permeability of propranolol or of caffeine, L-dopa, or dopamine. However, the use of cosolvent increased the permeability of carbamazepine. In the case of L-dopa and dopamine, their permeability in the presence of 20% 1-propanol was still zero. This result indicates that in this condition (20% 1-propanol) the phospholipid remained stable.

The PAMPA was performed on our nine-peptide library to study the relationship between chain length and permeability of these compounds. In all experiments propranolol (a well-known β-adrenergic receptor blocker) and carbamazepine (an anticonvulsant and mood-stabilizing drug used mainly in the treatment of epilepsy and bipolar effective disorder) were used as positive controls.

In this series of *N*-MePhe oligomers, increasing peptide chain length caused increased permeability up to the peptide with four

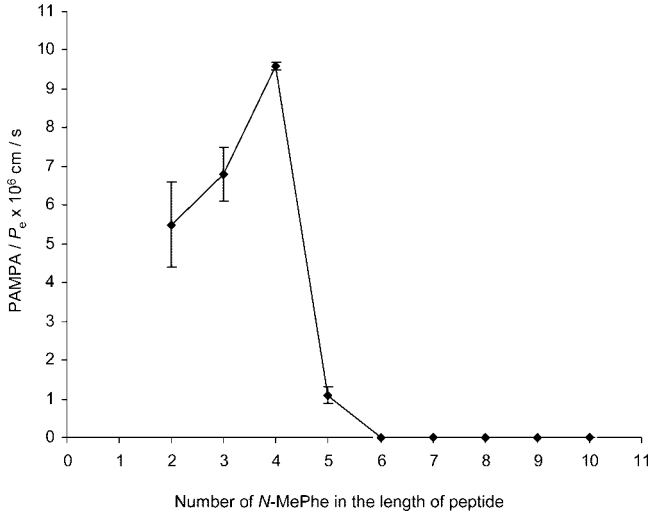

Figure 1. Relationship between PAMPA P_e and peptide length. Data are expressed as the mean ± SD.

Table 2. Percentage of Transport after 4 h, Effective Permeability (P_e) in the PAMPA, and k_{IAM} of Peptides Ac-(*N*-MePhe)$_n$-CONH$_2$ (n = 2−10) and Control Compounds (Propranolol, Carbamazepine)[a]

compd	P_e ($\times 10^6$), cm/s	transport (4 h), %	k_{IAM}
propranolol	7.4	13.5	1.9
carbamazepine	10.4	17.7	2.1
Ac-(*N*-MePhe)$_2$-CONH$_2$	5.5 ± 1.1	10.3 ± 1.8	3.2
Ac-(*N*-MePhe)$_3$-CONH$_2$	6.8 ± 0.7	12.5 ± 1.1	17.7
Ac-(*N*-MePhe)$_4$-CONH$_2$	9.6 ± 0.1	16.7 ± 0.2	>128[b]
Ac-(*N*-MePhe)$_5$-CONH$_2$	1.1 ± 0.2	2.3 ± 0.5	>128[b]
Ac-(*N*-MePhe)$_6$-CONH$_2$	0	0	>128[b]
Ac-(*N*-MePhe)$_7$-CONH$_2$	0	0	>128[b]
Ac-(*N*-MePhe)$_8$-CONH$_2$	0	0	>128[b]
Ac-(*N*-MePhe)$_9$-CONH$_2$	0	0	>128[b]
Ac-(*N*-MePhe)$_{10}$-CONH$_2$	0	0	>128[b]

[a] Data are expressed as the mean ± SD. [b] Retention time higher than 60 min in the IAMC HPLC column.

N-MePhe residues but after that permeability dropped markedly (Figure 1). Peptide with four *N*-MePhe residues showed the greatest transport (16.7%), which was higher than that of Ac-(*N*-MePhe)$_3$-CONH$_2$ (12.5%), an in situ BBB-positive peptide,[21] and of propranolol (13.5%) (Table 2).

IAMC. IAMC is a model of interaction between compounds and a phospholipid. In this approach a phospholipid (phosphatidylcholine) is covalently attached to silica and it mimics a wide range of solute−membrane interactions include ionic, lipophilic, and hydrogen-bonding interactions, which can be combined under a parameter known as phospholipophilicity. This approach has been used to predict the solute partitioning into fluid liposome membrane,[55] rat small intestinal absorption, oral drug absorption in mice, drug permeability in caco-2 cells,[56] human skin permeability,[57] oral drug absorption in humans,[58] and the permeation of drugs across the BBB and to differentiate between CNS− and CNS+ compounds.[59,60] The capacity factor (k_{IAM}), calculated using the retention time of the test compound, is used to estimate the membrane partition coefficient. k_{IAM} has shown better correlation than log *P* (octanol/water partition coefficient), log $D_{7.4}$ (octanol/buffer distribution coefficient), and log k_w (octadecyl silica HPLC capacity factor) with liposome systems,[55] oral drug absorption in mice,[56] and in vivo assay.[60]

k_{IAM} was determined for the nine peptides and controls (Table 2). The increase in *N*-MePhe groups through this neutral series simultaneously increased the number of H-bonds (hydrophilicity) and the number of large hydrocarbon side chains (hydrophobicity). These two factors have opposite effects on retention in IAMC (phospholipophilicity) as well as on permeability. For the first three members of the peptide family, an increase in chain length induced higher retention in IAMC and higher PAMPA permeability; it indicates that hydrophobicity is a major interaction. These findings are consistent with those reported by Taillardat-Bertschinger et al.[61] The remaining peptides showed very high retention in IAMC and no permeability in PAMPA (except Ac-(*N*-MePhe)$_5$-CONH$_2$), probably because of their size or high lipophilicity.

Table 3. Percentage of Transport after 4 h, Effective Permeability (P_e) in the PAMPA, and k_{IAM} of Peptides X-(*N*-MePhe)$_4$-Y (X = Ac or H and Y = CONH$_2$ or COOH) and Control Compounds (Propranolol, Carbamazepine)[a]

compd	P_e ($\times 10^6$), cm/s	transport (4 h), %	k_{IAM}
propranolol	6.9	12.7	1.9
carbamazepine	10.6	18.1	2.1
H-(*N*-MePhe)$_4$-COOH	1.4 ± 0	2.8 ± 0	5.7
H-(*N*-MePhe)$_4$-CONH$_2$	7.4 ± 0.7	13.4 ± 1.1	67.8
Ac-(*N*-MePhe)$_4$-CONH$_2$	6.8 ± 1.3	12.7 ± 2.1	>128[b]
Ac-(*N*-MePhe)$_4$-COOH	0.9 ± 0.01	1.9 ± 0.4	12.6

[a] Data are expressed as the mean ± SD. [b] Retention time higher than 60 min in the IAMC HPLC column.

Evaluation of Terminal Groups. After optimizing peptide length, we evaluated the effect of terminal groups. For this purpose, four peptides with distinct terminal groups were synthesized and studied by PAMPA and IAMC (Table 3). The synthetic methodology was similar to that used for the previous library except the Sieber amide resin was replaced by a 2-chlorotrityl resin in order to obtain a carboxylic acid at the C-terminus. These four peptides at physiological pH (7.4) cover a broad range of species: zwitterionic ($^+$H$_2$*N*-MePhe-(*N*-MePhe)$_3$-COO$^-$), basic, or positively charged ($^+$H$_2$*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$), neutral (Ac-(*N*-MePhe)$_4$-CONH$_2$), and acidic or negatively charged (Ac-(*N*-MePhe)$_4$-COO$^-$). The effect of changing CONH$_2$ to COO$^-$ on the capacity factor and permeability was dramatic. Although for both functional groups three hydrogen bonds form with solvent water,[14] the carboxylic group made a stronger hydrogen bond than the amide group, perhaps because it is a charged group at pH 7.4 and therefore caused the peptide to have less interaction with the phospholipids and hence lower permeability (Table 3). Austin et al.[62] showed that positively charged amines penetrate membranes. In our case, zwitterionic species ($^+$H$_2$*N*-MePhe-(*N*-MePhe)$_3$-COO$^-$) showed a lower capacity factor in IAMC and lower permeability in PAMPA than those from positively charged species ($^+$H$_2$*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$). On the basis of these results, we can conclude that the interaction of anionic carboxylic acid in the C-terminus with solvent water is dominant compared to electrostatic interaction of N-terminus protonated amine group with polar groups of phospholipids. Acetylation in the N-terminus did not have a notable effect on permeability, whereas it improved lipophilicity. This increase in k_{IAM} demonstrates the importance of hydrophobic interaction for IAMC retention compared to the electrostatic one. Although $^+$H$_2$*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ and Ac-(*N*-MePhe)$_4$-CONH$_2$ showed almost the same permeabilities in the PAMPA, the peptide with acetyl as N-terminus was chosen because in IAMC it exhibited higher retention time, which implies higher phospholipophilicity. Furthermore, acetyl can act as a small cargo that mimics the situation in which a peptide carries a cargo (drug) through the BBB. These findings suggested that acetyl and amide groups were the best options for the N-terminus and C-terminus of our BBB-shuttle, respectively.

Effect of Amino Acid Replacement. To establish the effect of amino acid replacement on peptide permeability, a library

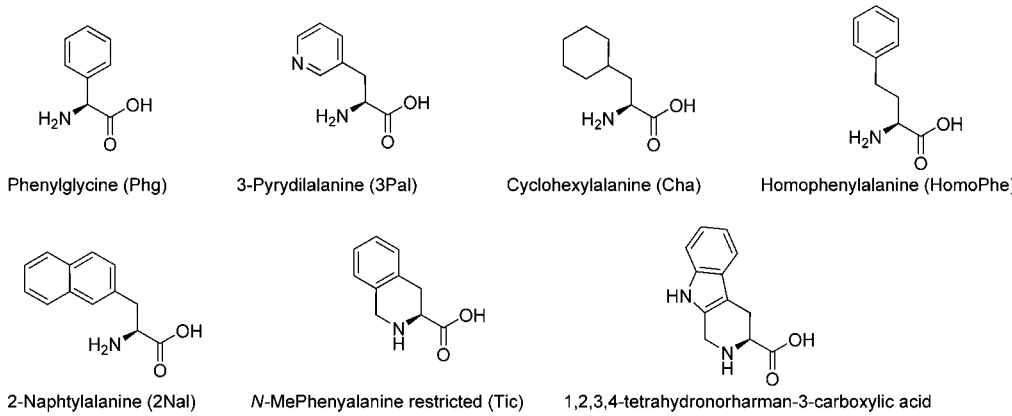

Figure 2. Structures of several unusual amino acids.

Table 4. Percentage of Transport after 4 h, Effective Permeability (P_e) in the PAMPA, and k_{IAM} of Peptides X-(*N*-MePhe)$_3$-CONH$_2$ (X = Diverse Amino Acids) and Control Compounds (Propranolol, Carbamazepine)

compd	P_e ($\times 10^6$), cm/s	transport (4 h), %	k_{IAM}
propranolol	7.9	16.6	1.9
carbamazepine	8	16.9	2.1
Ac-Phe-(*N*-MePhe)$_3$-CONH$_2$	2.3	4.9	43.4
Ac-*N*-MePhe -(*N*-MePhe)$_3$-CONH$_2$	6.4	13.4	>128[a]
Ac-Gly-(*N*-MePhe)$_3$-CONH$_2$	0.6	1.3	5.3
Ac-*N*-MeGly-(*N*-MePhe)$_3$-CONH$_2$	0.7	1.5	9.3
Ac-Phg-(*N*-MePhe)$_3$-CONH$_2$	0	0	25.9
Ac-*N*-MePhg-(*N*-MePhe)$_3$-CONH$_2$	0.7	1.5	>128[a]
Ac-Cha-(*N*-MePhe)$_3$-CONH$_2$	2.6	5.5	>128[a]
Ac-*N*-MeCha-(*N*-MePhe)$_3$-CONH$_2$	1.9	4	>128[a]
Ac-HomoPhe-(*N*-MePhe)$_3$-CONH$_2$	2.	4.3	>128[a]
Ac-*N*-MeHomoPhe-(*N*-MePhe)$_3$-CONH$_2$	1.8	3.7	>128[a]
Ac-2Nal-(*N*-MePhe)$_3$-CONH$_2$	3.1	6.6	>128[a]
Ac-*N*-Me2Nal-(*N*-MePhe)$_3$-CONH$_2$	1.3	2.8	>128[a]
H-Tic-(*N*-MePhe)$_3$-CONH$_2$	2.2	4.6	>128[a]
Ac-Tic-(*N*-MePhe)$_3$-CONH$_2$	2	4.3	>128[a]
Ac-Trp-(*N*-MePhe)$_3$-CONH$_2$	1.8	3.9	87.2
Ac-*N*-MeTrp-(*N*-MePhe)$_3$-CONH$_2$	1.4	2.9	>128[a]
Ac-Tyr-(*N*-MePhe)$_3$-CONH$_2$	1.1	2.3	10.8
H-1,2,3,4-tetrahydronorharman-3-carboxylic acid-(*N*-MePhe)$_3$-CONH$_2$	0.6	1.3	13.6
Ac-1,2,3,4-tetrahydronorharman-3-carboxylic acid-(*N*-MePhe)$_3$-CONH$_2$	1.5	3.2	24.6
Ac-3Pal-(*N*-MePhe)$_3$-CONH$_2$	1.2	2.5	6.3

[a] Retention time higher than 60 min in the IAMC HPLC column.

consisting of 20 peptides, which differ only in the N-terminal amino acid (4th), was designed and synthesized in solid phase. The amino acids chosen (except Gly) were structurally related to phenylalanine. We sought to study the effect of the following on permeability: phenyl ring modifications such as a larger aromatic ring (2Nal), a conformation restriction (Tic, 1,2,3,4-tetrahydronorharman-3-carboxylic acid), the presence of nitrogen (3Pal) and hydroxyl group (Tyr), side chain length (Phg, HomoPhe), and the lack of aromaticity (Cha) (Figure 2). For the sake of simplicity, the N-terminus amino acid position was selected for replacement.

The peptides were evaluated by PAMPA and IAMC (Table 4). From this library Ac-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ (13.4%), Ac-2Nal-(*N*-MePhe)$_3$-CONH$_2$ (6.6%), and Ac-Cha-(*N*-MePhe)$_3$-CONH$_2$ (5.5%) showed the best transport capacity after 4 h. The pyridine ring (3Pal, 2.5%) and phenol ring (Tyr, 2.3%) were less effective than the phenyl ring (Phe, 4.9%), probably because of the presence of nitrogen and the hydroxyl group, which increased the number of potential hydrogen bonds and decreased lipophilicity. In contrast, a larger aromatic ring (2Nal, 6.6%) or removal of aromaticity (Cha, 5.5%) increased permeability. The corresponding 2Nal and Cha N-methylated amino acids showed lower effectiveness. The addition of an extra methylene group to the phenylalanine (HomoPhe, 4.3%) proved better than removing the methylene group of phenylalanine (Phg, 0%). While Mahar et al.[41] showed that CNS drugs are less flexible than non-CNS drugs, recently Ballet et al.[63] reported that, in the case of two opioid peptides, rigidity is not a key factor for BBB permeation and does not affect lipophilicity either. In addition, Boguslavsky et al.[64] observed that cyclic analogues of enkephalin exhibit 3−7 times less permeation than corresponding acyclic peptides. In our study, while amino acid restriction (Tic) caused a decrease in permeation for *N*-MePhe (*N*-MePhe 13.4% vs Ac-Tic 4.3%), the less flexible tryptophan analogue (Ac-1,2,3,4-tetrahydronorharman-3-carboxylic acid) exhibited less phospholipophilicity but higher permeability (Ac-1,2,3,4-tetrahydronorharman-3-carboxylic acid 3.2% vs *N*-MeTrp 2.9%). All N-methylated amino acids demonstrated higher or equal retention in IAMC compared to corresponding unmethylated amino acids. However, this was not the case for PAMPA permeability, suggesting that other factors besides lipophilicity influence permeability.

Of all the N-methylated amino acids, *N*-MePhe showed the greatest transport capacity while Cha and 2Nal were the best unmethylated residues. From this library, we selected combinations of the three best amino acids, namely, Ac-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, Ac-Cha-(*N*-MePhe)$_3$-CONH$_2$, and Ac-2Nal-(*N*-MePhe)$_3$-CONH$_2$, as BBB-shuttles.

Stability Assay in Human Serum. One of the limitations of using peptides as therapeutic agents or drug carriers is their susceptibility to enzymatic cleavage. Therefore, several strategies, such as N-methylation of amide bond,[65,66] use of D-amino acids,[67,68] unnatural amino acids,[69,70] or β-amino acids,[71] cyclization,[72] and glycosylation,[73] have been applied to improve peptide stability against metabolic degradation.

The stability of our peptides in human serum was tested and compared to that of Ac-(Phe)$_4$-CONH$_2$. The peptides were analyzed by HPLC and HPLC−MS or MALDI. The data show a considerable enhancement of stability of the peptides in contact with enzymes because of the use of N-methylated or unusual amino acids. While only 6% of the parent peptide Ac-(Phe)$_4$-CONH$_2$ remained intact after 12 h, 91%, 57%, and 86% of Ac-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, Ac-Cha-(*N*-MePhe)$_3$-CONH$_2$, and Ac-2Nal-(*N*-MePhe)$_3$-CONH$_2$, respectively, remained stable within the same time period.

Cell Viability with MTT Assay. The effect of peptides on viability and proliferation of HeLa cells was studied via the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Figure 3). Three concentrations of peptide, 25, 50, and 100 μM, were added to HeLa cells and incubated for

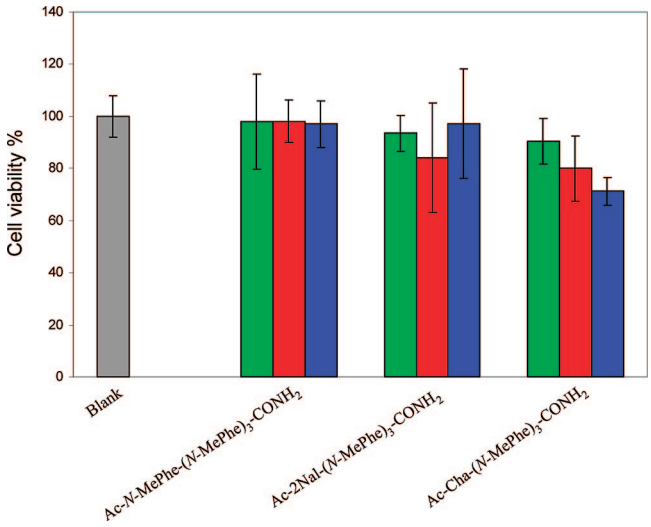

Figure 3. HeLa cell viability of blank and peptides at three concentrations after a 24 h of incubation. Green columns stand for 25 μM, red for 50 μM, and blue for 100 μM. Data are expressed as the mean ± SD.

Table 5. Percentage of Transport after 4 h, Effective Permeability (P_e) in the PAMPA, and k_{IAM} of L-Dopa, L-Dopa−BBB-Shuttles, and Control Compounds (Propranolol, Carbamazepine)[a]

compd	P_e ($\times 10^6$), cm/s	transport (4 h), %	k_{IAM}
propranolol	9.9	17.1	1.9
carbamazepine	10.6	18	2.1
L-dopa	0	0	0.3
L-dopa-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$	1.1 ± 0.1	2.4 ± 0.2	29.6
L-dopa-Cha-(*N*-MePhe)$_3$-CONH$_2$	0.7 ± 0.06	1.4 ± 0.09	>128[b]
L-dopa-2Nal-(*N*-MePhe)$_3$-CONH$_2$	0.3 ± 0.1	0.7 ± 0.2	102

[a] Data are expressed as the mean ± SD. [b] Retention time higher than 60 min in the IAMC HPLC column.

24 h at 37 °C. Ac-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ and Ac-2Nal-(*N*-MePhe)$_3$-CONH$_2$ not only were nontoxic to the cells but also did not influence normal cell proliferation. However, Ac-Cha-(*N*-MePhe)$_3$-CONH$_2$ at higher concentrations showed slight toxicity.

Application of Peptides as BBB-Shuttles. Strategies currently recommended to increase drug delivery to the brain include the use of nanoparticles, immunoliposomes, BBB-shuttles, compounds that are substrates for carrier-mediated transport, and peptide vectors. Among these, only BBB-shuttles carry drugs to the brain by passive diffusion, which is the main pathway for drug delivery to the CNS.[11,74–77]

Levodopa, a prodrug to dopamine that has been the most efficacious drug therapy for Parkinson's disease since 1967, was used as a cargo to evaluate the capacity of the three selected peptides to carry drugs through an artificial membrane. Levodopa can be transported to the brain by a large natural amino acid carrier, which is a saturable and competitive pathway.[78,79] To increase the uptake of levodopa, it was attached to the peptides using SPPS, and levodopa−peptide products were studied by PAMPA and IAMC (Table 5). By coupling levodopa to GSH (a tripeptide) through its carboxylic or amine groups to form an amide bond, Pinnen et al.[80] showed that levels of levodopa in rat plasma were higher compared to levels registered after administration of levodopa alone, while inside the brain levodopa was released from GSH sustainedly. Di Stefano et al.[81] and Gannazza et al.[82] observed that administration of dimeric levodopa derivatives with an amide bond increased levodopa and dopamine levels in rat brain more than the administration of levodopa alone. Theses results demonstrate that levodopa derivatization through an amide bond reduces the decarboxylation of levodopa to dopamine outside the brain, which is one of disadvantages of levodopa, thus decreasing the side effects of this drug. While inside the brain, this amide bond can be cleaved and free levodopa is released. On the basis of these observations, we coupled levodopa to the peptides through its carboxylic group to form an amide bond (Figure 4).

While levodopa alone did not show permeability in the PAMPA, our peptides carried this drug in this assay and exhibited great permeability (Table 5). Among the levodopa−peptides evaluated, levodopa-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$ showed the best permeation and was in the range of compounds with high permeability, similar to the situation in which peptides carry acetyl as a cargo (Table 4). The two other levodopa−peptides were considered medium-permeating compounds.[83] To demonstrate that the linkages between drug and carriers were not cleaved under assay conditions, the presence of levodopa−peptides in acceptor wells of the PAMPA plate were confirmed by both HPLC and MALDI-TOF mass spectrometry.

The capacity factors of levodopa and levodopa−peptides show that levodopa−shuttle products exhibited higher phospholipophilicity than levodopa alone. However, compared with their parent peptides, these levodopa−shuttles showed lower phospholipophilicity (except L-dopa-Cha-(*N*-MePhe)$_3$-CONH$_2$) and permeability (Tables 4 and 5), which could be attributed to an increase either in hydrogen bond number or MW.

Peptide Family Containing Phenylalanine. To observe the influence of N-methylation on peptide permeability and solubility and to compare with the results of the first library (Table 2), a similar peptide family, but using phenylalanine as amino acid, was synthesized (Ac-(Phe)$_n$-CONH$_2$ (n = 2, 4, 6, 8, 10)). From the synthetic point of view, only dipeptide and tetrapeptide were characterized by HPLC, HPLC−MS, MALDI-TOF MS, and HRMS. The hexapeptide was too insoluble in H_2O or/and MeCN to be examined by HPLC, and only the desired mass of peptide was identified by MALDI. The two remaining peptides with 8 and 10 Phe residues were not characterized by MALDI because of their low solubility in all the solvent mixtures assayed.

On first sight, this water insolubility was surprising and could be attributed to the major tendency of peptides to form H-bonds with each other instead of with solvent water, while peptides with *N*-MePhe residues are only H-bond acceptors and there is no H-bond formation between two molecules of the peptide.

Two soluble peptides (dipeptide and tetrapeptide) were examined by PAMPA and IAMC (Table 6). Neither were permeating in the PAMPA, and their capacity factors in IAMC indicated less membrane retention than corresponding N-methylated peptides (Tables 2 and 6). These results show that, at least for this series of compounds, N-methylation increased solubility, lipophilicity, and permeability. These results are consistent with previous studies by Chikhale et al.,[21] who observed that methylation of amide bonds in a series of peptides containing phenylalanine had a small effect on lipophilicity but increased permeability.

Permeability of Ac-(Phe)$_2$-CONH$_2$ in in vitro BBME cells and in situ rat brain perfusion were examined by Chikhale et al.[21] In the former the peptide showed high permeability (21.6 $\times$ 10^{-6} cm/s), while in the latter it had very low permeability (0.17 $\times$ 10^{-6} cm/s). The large difference between permeabilities in the two assays was attributed to paracellular flux in the in

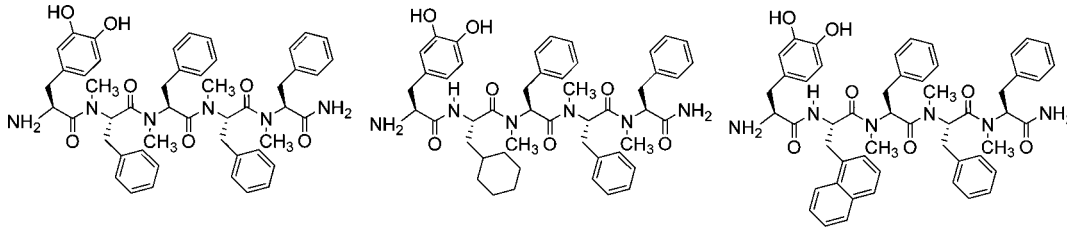

Figure 4. Structures of levodopa−peptides.

Table 6. Percentage of Transport after 4 h, Effective Permeability (P_e) in the PAMPA, and k_{IAM} of Peptides Ac-(Phe)$_n$-CONH$_2$ (n = 2, 4) and Control Compounds (Propranolol, Carbamazepine)

compd	P_e ($\times 10^6$), cm/s	transport (4 h), %	k_{IAM}
propranolol	7.9	16.6	1.9
carbamazepine	8	16.9	2.1
Ac-(Phe)$_2$-CONH$_2$	0	0	1.1
Ac-(Phe)$_4$-CONH$_2$	0	0	7.2

vitro model. In comparison, PAMPA permeability for this peptide (zero) was more similar to that registered in the in situ assay.

Conclusions

Here, we describe our research efforts to identify peptides with the capacity to carry drugs, which lack capacity to pass the BBB by themselves, into the brain by passive diffusion. For this purpose, several peptide libraries were designed and later synthesized in SPPS and assessed by PAMPA and IAMC.

Peptide length was first optimized, and a peptide with four *N*-MePhe residues was selected. Afterward the influence of the terminal groups of a peptide on its permeability was studied. In this phase, peptide with acetyl and amide groups as N-terminus and C-terminus respectively showed the best permeation. In the last step of optimization, the effectiveness of amino acid substitution was examined. Of the 20 peptides synthesized in this stage, Ac-*N*-MePhe-(*N*-MePhe)$_3$-CONH$_2$, Ac-Cha-(*N*-MePhe)$_3$-CONH$_2$, and Ac-2Nal-(*N*-MePhe)$_3$-CONH$_2$ showed better diffusion and were selected as BBB-shuttles. The use of N-methylated or unusual amino acids increased the stability of the peptides in human serum in comparison with the parent peptide. In addition, the MTT assay indicated that the peptides were not toxic to HeLa cells.

Finally, the three peptides showing greatest diffusion were coupled to levodopa to test their capacity to shuttle this drug in PAMPA. The peptides carried levodopa through the artificial membrane by passive diffusion and are therefore considered potential candidates as BBB-shuttles to transport drugs to 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). TBTU was purchased from Iris Biotech (Marktredwitz, Germany). PyBOP was supplied by Calbiochem-Novabiochem AG. DIEA, ninhydrin, and β-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 supplied by KaliChemie (Bad Wimpfen, Germany). 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). IAM column (10 mm × 4.6 mm, 12 μm, 300 Å, IAM.PC.DD2 column) was from 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 wavelength 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 wavelength 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.

General Protocols for Solid-Phase Synthesis. Syntheses were performed on a 100 μmol-scale/each, and 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[84] was used for the detection of solid-phase bound primary amines, while the De Clercq test[85] was used for secondary amines bound to solid-phase.

Initial Conditioning of Resin. The Sieber resin[86] 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).

The 2-chlorotrityl chloride resin was conditioned by swelling in DCM (15 min). Fmoc-*N*-MePhe-OH (0.7 equiv) was then attached to the resin with DIEA (7 equiv) in DCM (2 mL) for 1 h, without filtration. The remaining active positions were capped by methanol (resin methanol; 1 g, 0.8 mL) for 15 min. The resin was filtered and washed with DCM (5 × 30 s). Fmoc was removed, and filtrates were collected and measured by UV spectroscopy to determine the loading capacity (0.49 mmol/g).

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, an additional treatment with DBU, toluene, piperidine, 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, 218 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, and 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 Phenylalanine. N-Protected phenylalanine (4 equiv, 155 mg) and TBTU (4 equiv, 128.4 mg) were added sequentially to the resin in DMF (3 mL) followed by DIEA (8 equiv, 104 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 extent of coupling was checked by the Kaiser colorimetric assay. The peptide elongation continued by coupling the second amino acid and the following amino acid with the same procedure.

Amino Acid N-Alkylation. The N-methylation of the amino acid derivatives was performed using the method described by Miller.[35] 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 proceed to *o*-NBS removal, β-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.

Acetylation of N-Terminal Amino Acid. Acetylation was performed on solid phase using a standard protocol of Ac_2O (50 equiv) and DIEA (50 equiv) for 20 min.

Levodopa Coupling. Amine-protected levodopa (4 equiv, 168 mg), PyBOP (4 equiv, 218 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. The extent of coupling was checked by the De Clercq test or the Kaiser colorimetric assay.

Cleavage of the Peptides. Final amide or carboxylic acid 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) with 10 mL/min flow with the following solvents: solvent A, H_2O with 0.1% TFA; solvent B, MeCN with 0.05% TFA.

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

Parallel Artificial Membrane Permeability Assay (PAMPA). The PAMPA was used to determine the capacity of compounds to cross a model of the BBB by passive diffusion.[42] The effective permeability of the compounds was measured at an initial concentration of 200 μM. The buffer solution was prepared from a concentrated one and commercialized by pION, and the manufacturer's instructions were followed. The pH was adjusted to 7.4 using a 0.5 M NaOH solution. The compound of interest was dissolved in buffer solution 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. An amount of 4 μL of the mixture of phospholipids (20 mg/mL) in dodecane was added to the filter of each well, and 160 μL of buffer solution and 40 μL of 1-propanol were 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 an amount of 100 μL/each sample was injected in 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 be sure the compound had kept its integrity.

The phospholipid mixture used was a porcine polar brain lipid extract. Composition was 12.6% phosphatidylcholine (PC), 33.1% phosphatidylethanolamine (PE), 18.5% phosphatidylserine (PS), 4.1% phosphatidylinositol (PI), 0.8% phosphatidic acid, and 30.9% of other compounds. The percentage of transport after 4 h was calculated, and the effective permeability was calculated using eq 1:

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

where t is time (h), $C_A(t)$ is the compound concentration at the acceptor well at time t, and $C_D(t_0)$ is compound concentration in the donor well at 0 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 mm × 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 2,

$$k_{IAM} = (t_R - t_0)/t_0 \qquad (2)$$

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

Stability Assay in Human Serum. The peptide (200 μM) was dissolved in 300 μL of human serum and incubated at 37 °C on the orbital shaker (100 rpm). After the desired time, 300 μL of MeCN was added to the solution to precipitate the serum. The sample was then centrifuged (15 min, 10 000 rpm) at 4 °C, and then 150 μL of supernatant was taken and analyzed by HPLC. The presence of peptide was demonstrated by MALDI. Percentage of intact peptide was calculated by dividing the peak area of peptide at a given time to that of the peptide at time zero.

Cell Viability with MTT Assay. Cell toxicity of peptides was determined using a (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Hela cells were cultured (7000 cells/well) in a 96-well plate and incubated for 24 h. Incubation medium was then removed, and peptide solution in medium was added at concentrations of 25, 50, and 100 μM and incubated for 24 h at 37 °C. Given that the peptides were not completely soluble in medium, the peptide solutions were centrifuged before being added to cells. After 22 h, MTT was added to a final concentration of 0.5 mg/mL and incubated for 2 h more. The medium was then removed, and 200 μL of 2-propanol was added in order to dissolve the purple formazan produced. Absorbance was measured at 570 nm after 45 min. Cell viability percentages were determined by dividing the absorbance value of cells treated with peptide by that of untreated cells. The effect of peptides on cell proliferation was determined by comparing the number of cells in contact with

peptides after the 24 h incubation with those without peptides and before incubation.

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

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