Backbone Cyclic Peptidomimetic Melanocortin-4 Receptor Agonist as a Novel Orally Administrated Drug Lead for Treating Obesity

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The tetrapeptide sequence His-Phe-Arg-Trp, derived from melanocyte-stimulating hormone (αMSH) and its analogs, causes a decrease in food intake and elevates energy utilization upon binding to the melanocortin-4 receptor (MC4R). To utilize this sequence as an effective agent for treating obesity, we improved its metabolic stability and intestinal permeability by synthesizing a library of backbone cyclic peptidomimetic derivatives. One analog, peptide **1** (BL3020–1), was selected according to its selectivity in activating the MC4R, its favorable transcellular penetration through enterocytes and its enhanced intestinal metabolic stability. This peptide was detected in the brain following oral administration to rats. A single oral dose of 0.5 mg/kg in mice led to reduced food consumption (up to 48% vs the control group) that lasted for 5 h. Repetitive once daily oral dosing (0.5 mg/kg/day) for 12 days reduced weight gain. Backbone cyclization was shown to produce a potential drug lead for treating obesity.

Introduction

Obesity is a major health problem in the developed world and is a high risk factor in many diseases, including diabetes type II, high blood pressure, cardiovascular and respiratory diseases, and cancer, which result in a significant reduction in life span and quality.[1]

According to the NHANES (National Health and Nutrition Examination Survey) conducted in 2003–2004, 17.1% of the children and adolescents aged 2 to 19 years in the United States were overweight and 32.2% of the adults aged 20 years or older were obese.[2] Although the United States has the highest prevalence of obesity among the developed nations, it is not alone in terms of trends. Increases in the prevalence of overweight and obese children and adults have been observed throughout the world.

Weight loss is always difficult to achieve through changes in life style and currently licensed antiobesity drug treatments such as orlistat and sibutramine, if tolerated, only achieve modest weight loss. Therefore, there is a need to identify more potent pharmacological targets.

The pharmaco-therapeutic approach for treating obesity has been to regulate the biochemical pathways that control food consumption and metabolic balance in the body. A key endocrine system that controls these physiological processes is the melanocortin pathway,[3] which includes binding the catabolic neuropeptide melanocyte stimulating hormone (αMSH[a]) to the melanocortin 4 receptor (MC4R). MC4R agonists decrease food intake and elevate energy utilization[3] such that they could be used for treating obesity. For example, a study performed with melatonan II (MTII), a commercially available peptide-based MC4R agonist, administered intracerebroventricularly for four weeks, resulted in a 40% decrease in rat weight.[4]

The effective role of a MC4 agonist in obesity therapy was also demonstrated in humans. Intravenous (iv) administration of the melanocortin sequence MSH/ACTH (4–10) to normal-weight human subjects for six weeks decreased body fat by 1.7 kg.[5] The sequence Phe-DPhe-Arg-Trp, a potent MC4R agonist,[6] was used in this study as the stem peptide to achieve an effective agent for treating obesity.[7,8] MC4R peptide agonist was found to decrease body fat in humans,[9] however, a major drawback of current MC4R agonist peptides is that they can not be taken orally due to poor bioavailability. Oral administration is the most convenient mode of drug intake and therefore is favored for chronic treatment to preserve proper patient compliance. The

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[a] Abbreviations: The abbreviations for amino acids are according to the IUPAC-IUB Commission of Biochemical Nomenclature, http://www.chem.qmul.ac.uk/iupac/aminoacid. The following abbreviations are used throughout the text: ACN, acetonitrile; Alloc, allyloxycarbonyl; BBMVs, brush border membrane vesicles; ; Boc, *t*-butoxycarbonyl; BTC, bis(trichloromethyl)carbonate; Bri, bridge unit; COSY, correlation spectroscopy; DCM, dichloromethane; DIEA, *N*,*N*-diisopropylethylamine; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; EtOAc, ethyl acetate; ESI, electrospray ionization; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, 9-fluorenylmethyloxycarbonyl-*N*-hydroxysuccinimide; HBTU, (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate); HOBt, 1-hydroxybenzotriazole; iv, intravenous; MALDI, matrix assisted laser desorption ionization; MBHA, methylbenzhydrylamine; MC4R, melanocortin-4 receptor; MCR, melanocortin receptor; αMSH, melanocyte stimulating hormone; NMP, 1-methyl-2-pyrrolidinone; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; P_{app}, permeability apparent; po, per oral; ROESY, rotating frame Overhauser effect spectroscopy; SPPS, solid phase peptide synthesis; TBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium tetrafluoroborate; tBu, *tert*-butyl; TIS, triisopropylsilane; TLC, thin layer chromatography; TOCSY, total correlation spectrometry.

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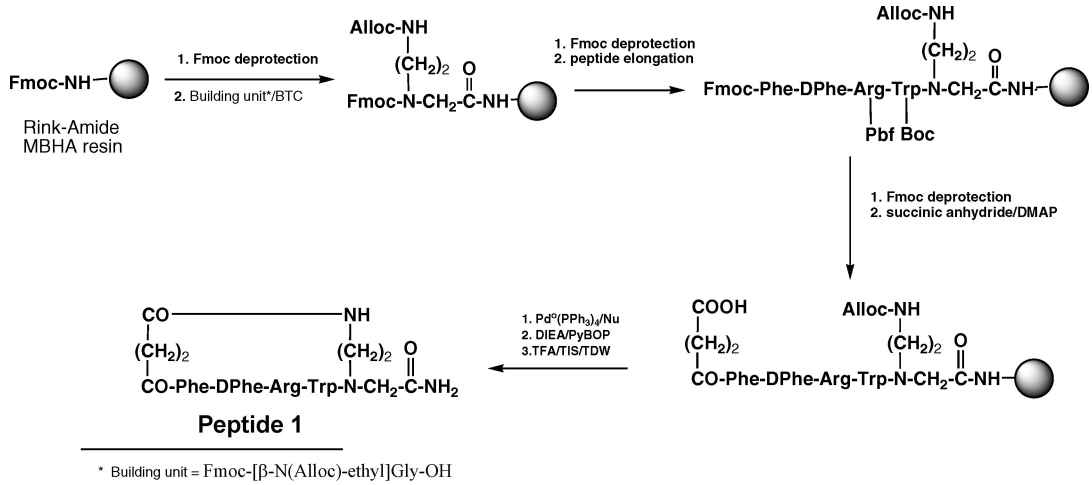


Figure 1. Scheme of peptide 1 synthesis.

generally poor oral bioavailability of peptides is due to extensive intestinal metabolic degradation, mainly by the intestinal brush border enzymes and poor intestinal permeability.[10]

In this work we assessed the effect of backbone cyclization on the MC4R agonist as a potential method to improve oral bioavailability while enabling brain availability and maintaining high affinity and selectivity at the receptor level.

Backbone cyclization[11,12] is a general method by which conformational constraint is imposed on peptides.[13] In backbone cyclization, a peptidomimetic is formed by covalently interconnecting atoms in the backbone (N and/or C) of a target linear peptide to form a ring. The advantage of backbone cyclization over other modes of peptide cyclization is that cyclization is achieved mainly using backbone atoms and not side chains that are essential for biological activity. This method has been shown to dramatically enhance the metabolic stability of peptides in serum.[12] In addition, this method has been shown to improve the pharmacological selectivity of a given peptide, as demonstrated for substance P[12] and somatostatin analogs.[14] It should be noted that the specific selectivity of the melanocortin agonist to MC4R is important to obtain the antiobesity activity while avoiding adverse effects caused by activation of other subtypes of MCR.

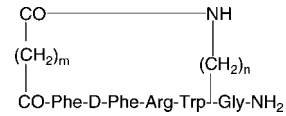
The aim of this study was to generate a selective, highly potent, MC4R agonist that is orally and brain available and can serve as an antiobesity drug lead.

Results and Discussion

Chemistry. A library of 16 backbone cyclic pentapeptides (called the BL3020 library[15]) was synthesized based on the parent peptide Phe-D-Phe-Arg-Trp-Gly NH_2, which was found to have high potency in activating the MC4R.[6] The parent peptide, Phe-D-Phe-Arg-Trp-Gly NH_2, of the BL3020 library was cyclized by a bridge that connects the N-terminus to the Nα of the C-terminal Gly building unit by a dicarboxylic acid spacer. The synthesis of **1** (BL3020-1) is depicted in Figure 1. The building unit Fmoc-[β-N(Alloc)-ethyl]Gly OH, and other building units used for the preparation of the library, were prepared as previously described.[16] All the peptides in the library bear the parent sequence but differ from each other in their ring size and ring chemistry (Table 1 shows selected members of the library). The peptides were studied for MC4R binding, MC receptor activation, and selectivity, as well as in vitro intestinal permeability and resistance to intestinal metabolic degradation.

Metabolic Stability of the Cyclic Peptide Analogs. The metabolic stability of members of the peptide library in the intestine was compared to that of the linear peptide (Figure 2). While the concentration of the linear peptide was reduced to nearly 40% of the initial concentration after 40 min of incubation with the brush border membrane vesicles (BBMVs), the cyclic peptides **1** and **2** (BL3020-15) were stable toward enzymatic degradation and showed less than a 5% reduction in the initial

Table 1. Structure of Selected Members of the Peptide Library[a]



peptide	*m*	*n*	ring size (number of atoms)
1	2	2	20
2	4	3	23
3	[b]	2	20
4	2	3	21
5	3	2	21

[a] The peptides in the library differ in their ring size (e.g., peptides **1** and **2**) and their ring chemistry (e.g., peptides **4** and **5** or peptides **1** and **3**). [b] The succinyl spacer ($m = 2$) of peptide **1** was replaced by *o*-phtaloyl spacer. The structure of peptide **3** is shown in Figure 3.

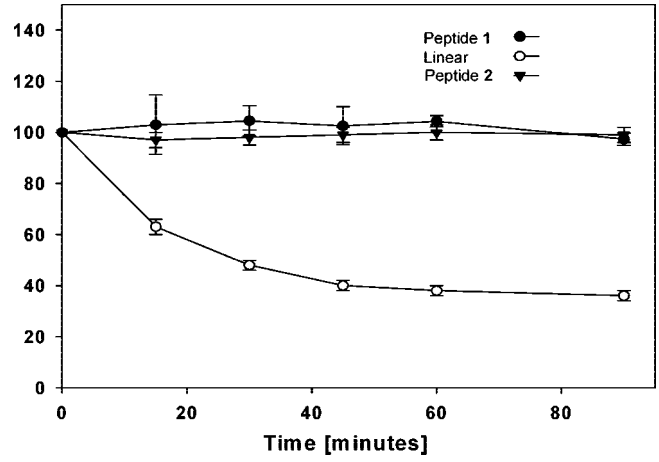


Figure 2. Metabolic stability of cyclic peptides **1** and **2** and the linear analog. The tested molecules were mixed with purified BBMVs and incubated at 37 °C for 90 min. Duplicate samples were taken at time 0 and after 15, 30, 45, 60, and 90 min. The samples were diluted 1:1 with ice-cold acetonitrile, centrifuged (7500 *g*, 10 min, 4 °C) and transferred to analysis, SD < 15% (peptide **1** ●, peptide **2** ▼, linear tetra-peptide analog ○).

Table 2. Summary of Affinity (IC_{50}) and Functional Activity (EC_{50}) at the Mouse Melanocortin Receptors[a]

		stimulation/potent activation (EC_{50}, nM)			
	binding to MC4R (IC_{50}, nM)	mMC1R	mMC3R	mMC4R	mMC5R
NDP-MSH	NT[b]	0.008 ± 0.001	0.063 ± 0.004	0.097 ± 0.016	0.078 ± 0.008
1	90	64 ± 14	770 ± 175	4.0 ± 0.6	7.0 ± 0.4
2	60	240 ± 41	7750 ± 1960	5.0 ± 0.08	26 ± 17
3	100	550 ± 97	20500 ± 2100	150 ± 22	390 ± 120
4	60	28 ± 12	1260 ± 385	4 ± 2	2 ± 1
5	NT	120 ± 28	1460 ± 145	18 ± 4	20 ± 4

[a] The affinities (half maximal inhibitory concentration IC_{50}, nM) and receptor functional activation (EC_{50}, nM) of the backbone cyclic peptides to the melanocortin-4 receptor. The efficacy was 100% (maximal stimulation relative to the NDP-MSH control) for all compounds tested. [b] NT = not tested.

concentration following 90 min of enzymatic reaction (Figure 2). The capability of the peptides to withstand the enzymatic activity of various peptidases located in the gut lumen and the intestinal brush border is a major factor in the oral bioavailability of peptides. Because much of the enzymatic digestion takes place at the surface of the small intestinal epithelial cells, there is usually a significant reduction in the ability of intact peptides to reach the systemic circulation after reaching the intestinal milieu. For tetra- and higher amino acid peptides, more than 90% of the proteolytic activity occurs in the brush border membrane, whereas for tripeptides 10–60% and for dipeptides only 10% of the proteolytic activity occurs in the brush border membrane.[17] These results are in agreement with previous reports from our laboratory that have demonstrated significant metabolic stability of backbone cyclic peptides in various media such as serum and kidney homogenate.[12,14]

Intestinal Permeability. In vitro permeability studies of the BL3020 library through Caco-2 monolayers resulted in permeability coefficient values of the cyclic peptides that were significantly higher than permeability coefficients (P_{app}) obtained for mannitol and atenolol, markers of paracellular transport, and only slightly smaller than P_{app} of propranolol, a marker for transcellular transport (Table 3). The permeability of the linear equivalent peptide was higher than atenolol (12×10^{-6} and 8.5×10^{-6} cm/sec, respectively); however, it was 50–60% lower than that of the cyclic peptides. The permeability of active compounds through Caco-2 monolayer (derived from a human colorectal adenocarcinoma) is an established model to determine intestinal permeability[18] and was also used to predict permeability of molecules through the blood−brain barrier (BBB).[19] The most exciting finding achieved in this work was that the high permeability coefficient of the tested analogs was similar to that of propranolol, a molecule that is known for its ability to traverse the intestinal wall via the transcellular pathway, indicating that most of the examined library analogs are highly permeable compounds.[20] The significant superior permeability in comparison to mannitol and atenolol is another indication that the mechanism of intestinal permeability is by a transcellular mechanism. It should be noted that this finding is unique in view of the tendency of many peptides to permeate the intestinal wall via the paracellular route.[21] Furthermore, backbone cyclization increased permeability relative to the linear analogue (Table 3). This is probably due to the conformational constraints imposed by cyclization, the elevated lipophilicity, and the reduced hydrogen-bonding potential characteristic of the cyclic peptides.[10] The transport of **1** from the apical side of the membrane to basolateral side (A to B) was compared to the transport from the basolateral to the apical (B to A) using Caco-2 cells. The transport from A to B was found to be 2-fold higher than the opposite direction.

This data suggest that there is an involvement of an active mechanism in the transport of the peptide. Further investigation of the underlying transport mechanism of this peptide will be performed to characterize the transport mechanism of **1**.

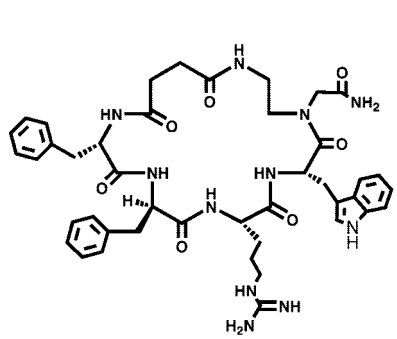
Table 3. Permeability coefficient (P_{app}) of Cyclic Peptides Compared to the Linear Analog and to Known Standards[a]

compound	P_{app} (cm/sec) × 10^6 ± SEM
mannitol	2.05 ± 0.8
atenolol	8.5 ± 0.4
propranolol	25 ± 2.3
linear	12 ± 0.98
1	17.8 ± 1.37
2	19 ± 5.5
4	18.8 ± 4.39
5	21 ± 3.6

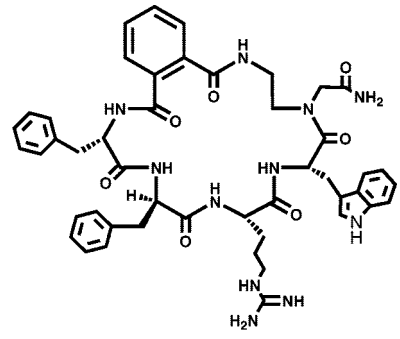
[a] Caco-2 cell monolayers were incubated with the tested molecules at 37 °C, added to the apical side, and detected at the basolateral side for 150 min; $n \geq 3 \pm$ SEM.

Ligand–Receptor Interactions and Agonist Activity at the Melanocortin Receptors. Following the stability and in vitro permeability studies, the peptides of the library were analyzed for biological activity in binding and activating the melanocortin receptors (Table 2). The changes in the ring size and chemistry led to observed changes in binding and potency of activating of the melanocortin receptors and, hence, the selectivity of the peptides to the different MC4s. The binding affinity values, IC_{50}, of the various backbone cyclic peptides to the MC4R receptors are given in Table 2. The binding assays indicate that most of the peptides had high affinity to the MC4R. Peptide **2** had the highest affinity, with an IC_{50} value of 60 nM. The IC_{50} value of peptide **1** reached 90 nM, and the IC_{50} value of peptide **3** (BL3020-16) was 100 nM. Because the IC_{50} values do not indicate whether the binding generates an agonist or antagonist effect, the potency of activating various melanocortin receptors was investigated (Table 2). The peptides were found to have diverse potency. For example, peptide **4** (BL3020-8) exhibited a similar potency to MC4R as that of **1** and increased potency by 2- and 4-fold toward MC1R and MC5R, respectively. The stimulation/agonist potency of MC4R was in the range of 4.0–18.5 nM for all library peptides (except for peptide **3**, which was found to be significantly less potent on all tested melanocortin receptors). The high affinity that was detected gave the first indication that the chemical modifications made did not damage the biological activity of the peptides. The results confirm that most of these peptides range from 80- to 1550-fold selective for the MC4R versus the MC3R.

Table 2 shows the selectivity and specificity of cyclic peptides from the library. While all the library components share the same sequence, the size and chemistry of the ring moiety differ, thereby affecting their binding affinity and potency in activating the melanocortin receptors. The conformational constraint achieved by backbone cyclization produced peptides with a much smaller and more defined conformational space than their linear counterparts. Moreover, the limited conformational space of each peptide in the library is different, hence, some of the



Peptide **1**



Peptide **3**

Figure 3. Structure of the backbone cyclic peptides **1** and **3**.

members of such libraries could become inactive because they cannot adopt the bioactive conformation of the MCRs. However, peptides that can conform to the bioactive structure are likely to be very potent and have all the pharmacological advantages of cyclic peptides.[22] Backbone cyclization affected both specificity and selectivity toward the various melanocortin receptors, allowing us to determine which peptide is a selective agonist that activates the target receptor, MC4R, with maximal specificity and minimal side effects.

Selection of the Lead Compound from the Peptide Library. The metabolic stability, permeability, and functional agonist activity assay of the library performed on the mouse melanocortin receptors indicated that the backbone cyclic peptide **1** (Figure 3) is the best candidate for a lead compound: it selectively activated the MC4R, it was metabolically stable, and it had high intestinal permeability. Therefore, it was selected for further characterization and its pharmacokinetic behavior was investigated.

NMR Analysis of 1. All protons were accounted for except the aromatic region, which was partially unresolved due to overlap. All the $H\alpha_i-HN_{i+1}$ connectivities were clearly seen. A strong ROE/NOE peak was observed between the Hβs and Hδs of each phenylalanine, giving the connectivity of the respective aromatic moieties. The HN^{Bri} of the bridge unit gave a unique triplet in the amide region of the spectrum and, thus, its identity. An interaction between $H\alpha^{Bri}$ and $HN^{Phe\text{-}1}$, together with the interaction of $H\delta^{Bri}$ to $H\alpha^{Trp\text{-}4}$, gave the direction of the bridge moiety. A COSY peak between $H\alpha^{Bri}$ and $H\beta^{Bri}$ and a strong ROE interaction between $H\beta^{Bri}$ and HN^{Bri} gave the identity and orientation of this segment of the bridge. TOCSY and COSY peaks between HN^{Bri}, $H\gamma^{Bri}$, and $H\delta^{Bri}$ identified this portion of the bridge unit. There were two conformations evident in the spectra as is common among N-alkylated cyclic peptides.[23] The ratio of conformers was 1:2 in the 1D spectrum. Chemical exchange during the time of the measurement was evident from the inverted exchange peaks in the ROESY spectra. The difference between conformations is due to the dihedral angle between the tertiary nitrogen and the carbonyl carbon of Trp^4 ($H\alpha^{Gly\text{-}5}$, $N^{Gly\text{-}5}$, $C^{Trp\text{-}4}$, $O^{Trp\text{-}4}$); this angle is 0° in the main conformation and 180° in the minor conformation. The main conformation is supported by ROE peaks between $H\alpha^{Trp\text{-}4}$ and $H\delta^{Bri}$ (or $H9^{Bri}$ in BBC12) and the lack of a ROE signal between $H\alpha^{Trp\text{-}4}$ and $H\alpha^{Gly\text{-}5}$. The $H\alpha^{Trp\text{-}4}$ of the minor conformation in most cyclic peptides overlaps the water signal, except for the case of BBC12, where the conformation of the minor conformation is inferred from the clear NOE interaction observed between $H\alpha^{Trp\text{-}5}$ and $H\alpha^{Gly\text{-}6}$ and the lack of a strong $H\alpha^{Trp\text{-}5}$ to $H9^{Bri}$ NOE. The $H\alpha^{Trp\text{-}4}$ of the minor conformation overlaps the water signal and, therefore, its conformation is inferred from similar peptides in other solvents (unpublished results). The proton chemical shift values of peptide **1** are summarized in Table 4.

Table 4. NMR Characterization of Peptide 1; Proton Chemical Shifts (ppm) of Peptide **1** Main (Top) and Minor (Bottom) Conformations

residues	HN	Hα	Hβ	others
		main conformation		
Bri^{0*}	7.73	2.39	2.31	δCH_2 2.82, 3.54
				γCH_2 3.14, 3.28
Phe^1	8.14	4.26	2.93	δCH_2 7.16
				ϵCH_2 UR[a]
D-Phe^2	7.41	4.51	2.83, 2.88	δCH_2 7.16
				ϵCH_2 UR
Arg^3	7.60	4.20	1.52, 1.73	γCH_2 1.36
				δCH_2 3.09
				HN 7.60
Trp^4	8.26	4.89	3.23	Hδ1 7.25
				Hϵ1 10.05
				Hϵ3 7.58
				Hη2 UR
				Hζ2 7.48
				Hζ3 UR
Gly^5		3.75, 4.04		NH_2 6.89, 7.13
		minor conformation		
Bri^{0*}	7.54	2.51, 2.58	2.38, 2.46	δCH_2 3.80
				γCH_2 3.23, 3.40
Phe^1	7.96	4.50	2.98	δCH_2 7.14
				ϵCH_2 UR
D-Phe^2	8.11	4.42	2.85	δCH_2 7.09
				ϵCH_2 UR
Arg^3	7.84	4.15	1.30, 1.51	γCH_2 0.96, 1.08
				δCH_2 2.92
				HN 7.0
Trp^4	7.89	4.73	3.21	Hδ1 7.25
				Hϵ1 10.06
				Hϵ3 7.64
				Hη2 UR
				Hζ2 7.48
				Hζ3 UR
Gly^5		4.01, 4.28		NH_2 7.57

[a] UR unresolved.

Pharmacokinetic Parameters following iv and po Administration. The PK of peptide **1** was studied following iv and po (dissolved in water, 1 mg/kg and 10 mg/kg, respectively) administration to rats. Analyzing the results produced several pharmacokinetic parameters, including the peptide half-life in the body (>105 min), the AUC value (24,980 min·ng/ml), and the volume of distribution at steady state (V_{ss}, 2.1 L/kg). Measured C_{max} values were 977 ± 97 (iv) and 202 ± 39 ng/ml (po). The T_{max} for iv was at 5 min and at 37 ± 10 min following po administration. The relatively high volume of distribution provides an indirect indication of the ability of **1** to cross biological membranes such as the intestine and the BBB.[24] The pharmacokinetic findings indicate a relatively high bioavailability of 8.5%.

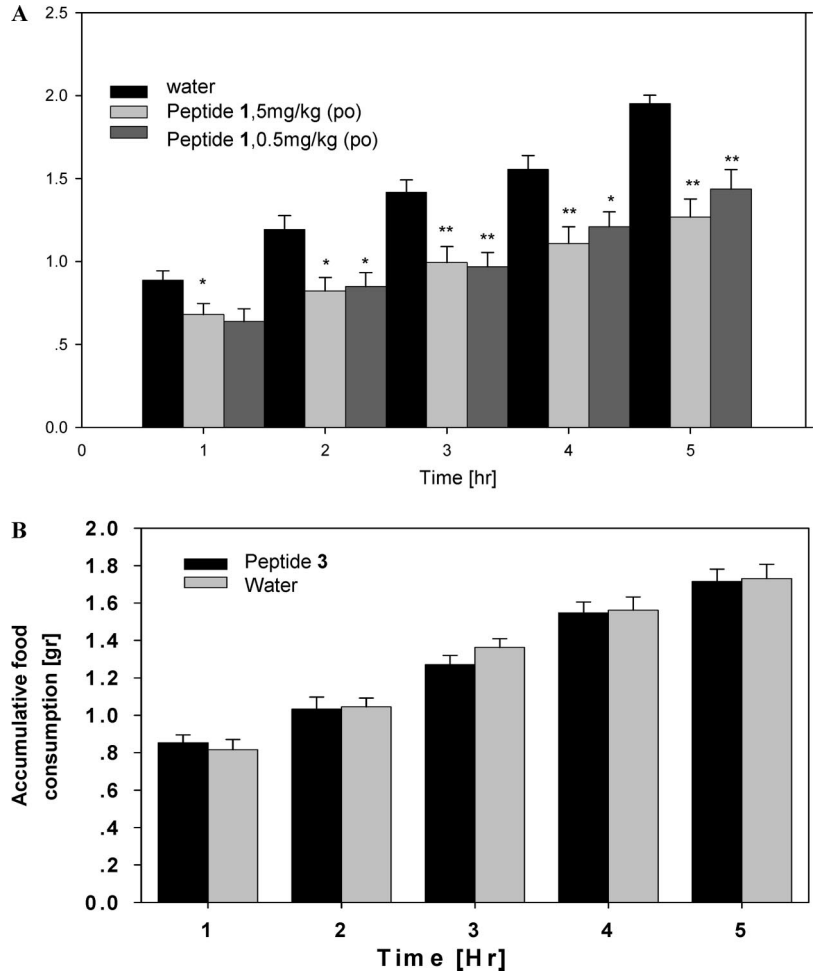


Figure 4. Short time effect of MC4R agonists on mice food consumption following po administration. Following fasting for 16 h, ICR:Hsd (CD-1) male mice ($n = 24$) were subjected to a single oral gavage (po, 5 mL/kg) of the peptide (100 μg/ml) or vehicle (water). Immediately after administration, fixed food doses were added and reweighed after 1, 2, 3, 4, and 5 h. (A) Black bars, control; gray bars, peptide **1**. (B) Black bars, control; gray bars, peptide **3**. Data are expressed as the mean ± SEM. Statistical analysis made by RM ANOVA with post hoc multiple comparisons using Tukey's test: $*P < 0.05$; $**P < 0.01$.

In Vivo Studies by Oral Administration. In vivo studies in mice showed reduced food consumption following oral administration of **1**, as depicted in Figure 4A. Reduced food consumption was already apparent 1 h after a single oral administration. The decrease in food consumption reached 46–48% in comparison to the untreated (control) group. No abnormal physiological or behavioral signs were observed during the assay. A parallel study was performed with peptide **3**, another member of the library that was found to be nonspecific and less potent than peptide **1** in MC4R stimulation or potent activation (Table 2). In this study no reduction in food consumption was observed up to 5 h postoral administration of peptide **3** (Figure 4B).

Following the observed acute effect of peptide **1** on food consumption, the effect of longitude repetitive once daily oral administration was tested in order to assess the effect on weight increase. As depicted in Figure 5, oral administration of peptide **1** resulted in inhibition of weight gain compared to the untreated animal group. While there is a consistent rise in weight gained by the control group, reaching over 7.5% after 10 days of treatment, the increase in weight in mice treated with peptide **1** was inhibited, gaining only 2.8% compared to their original weight on day 0.

Brain Bioavailability. The site of action of αMSH and other MCR agonists is within the hypothalamus.[25,26] Therefore, in addition to the requirement of intestinal metabolic stability and bioavailability, the ability to penetrate the BBB is of interest. The concentrations of peptide **1** in the brain determined 8 h post iv and po administration of 10 mg/kg were 107.3 ± 14.2 ng/mg and 4.7 ± 2.5 ng/mg, respectively. This provides direct evidence for the ability of peptide **1** to reach the brain tissue following oral administration.

Biological Activity of Peptide 1. The main concern in the process of improving oral bioavailability of peptides by using structural and conformational modifications is the loss of biological activity. Although it was demonstrated that the backbone cyclic peptides were found to preserve their in vitro binding and potent activation of the MC4R as agonists, further investigation of the in vivo effect was required. The effect of acute per oral administration on the food consumption (shown in Figure 4) provided proof of our concept that backbone cyclization is a practical means to obtain an active, orally available peptide that can be used for treating obesity.

It should be noted that the effect of both doses used on food consumption lasted for at least 5 h post oral administration. The elimination half-life (≈100 min in rats) suggests that the effect of the peptide on food consumption follows the indirect pharmacodynamic concept.[27]

The effect of repetitive once daily dosing of peptide **1** on inhibiting weight gain corroborated the concept of using the

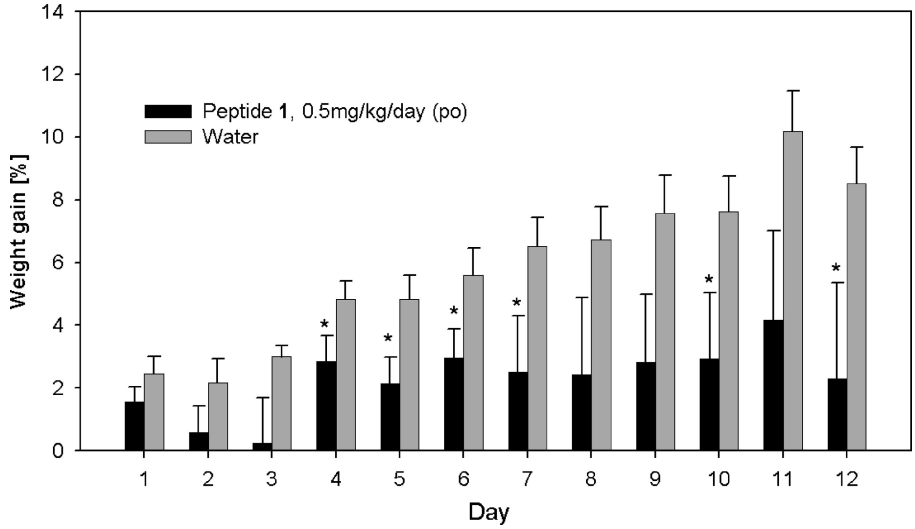


Figure 5. The effect of peptide **1** on mice weight gain after repeated po administration. Following daily weighing, mice ($n = 9$) were subjected to daily oral gavage (po, 5 mL/kg) of peptide **1** (100 μg/ml) or vehicle (water). Black bars, control; Gray bars, peptide **1**. Data are expressed as the mean ± SEM. Statistical analysis made by student's *t*-test: $^*P < 0.05$.

backbone cyclic MC4R analog as a potential novel orally available antiobesity drug.

Conclusions

The results of this study indicate that by utilizing backbone cyclization it is possible to synthesize bioactive peptides that are stable in the intestinal milieu and cross the intestinal wall, thus providing enhanced oral bioavailability. The current knowledge regarding the role of MC4R in regulating appetite, energy homeostasis and neuroendocrine function, coupled with an advanced peptide chemistry approach has resulted in the synthesis of a promising novel MC4R agonist lead for obesity therapy.

Methods

Chemistry. General. Protected amino acids, 9-fluorenylmethyloxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu), bromo-tris-pyrrolidone-phosphonium hexafluorophosphate (PyBrop), Rink amide methylbenzhydrylamine (MBHA) polystyrene resins, organic and supports for solid phase peptide synthesis (SPPS) were purchased from Nova Biochemicals (Laufelfingen, Switzerland). Bis(trichloromethyl)carbonate (BTC) was purchased from Lancaster (Lancashire, England). Glyoxylic acid, 1,2-diaminoethane, 1,3-diaminopropane, and 1,4-diaminobutane were purchased from Merck (Darmstadt, Germany), and tetrakis (triphenylphosphine) palladium(0) was purchased from ACROS (Geel, Belgium).

Nuclear magnetic resonance (NMR) spectra during synthesis were recorded on a Bruker AMX 300 MHz spectrometer. Mass spectra were performed on a Finnigan LCQ DUO ion trap mass spectrometer. Thin layer chromatography (TLC) was performed on Merck F245 60 silica gel plates (Darmstadt, Germany). HPLC analysis was performed using a Vydac analytical RP column (C18, 4.6 × 250 mm, catalog number 201TP54) and was carried out on a Merck-Hitachi L-7100 pump and a Merck-Hitachi L-7400 variable wavelength detector operating at 215 nm. The mobile phase consisted of a gradient system, with solvent A corresponding to water with 0.1% TFA and solvent B corresponding to acetonitrile (ACN) with 0.1% TFA. The mobile phase started with 95% A from 0 to 5 min followed by a linear gradient from 5% B to 95% B from 5 to 55 min. The gradient remained at 95% B for an additional 5 min and then was reduced to 95% A and 5% B from 60 to 65 min. The gradient remained at 95% A for additional 5 min to achieve column equilibration. The flow rate of the mobile phase was 1 mL/min. Peptide purification was performed by reversed phase HPLC (RP-HPLC; on a L-6200A pump, Merck-Hitachi, Japan) using a Vydac preparative RP column (C8, 22 × 250 mm, catalog number 218TP1022). The flow rate of the mobile phase was 9 mL/min. All preparative HPLC runs were carried out using a gradient system with solvent A corresponding to water with 0.1% TFA and solvent B corresponding to ACN with 0.1% TFA. MS characterization was performed on a Voyager-DE PRO Biospectrometry workstation using matrix assisted laser desorption ionization (MALDI) technology in the positive mode.

Solid Phase Peptide Synthesis of Peptide 1. The synthesis was performed in a reaction vessel equipped with a sintered glass bottom, following general Fmoc chemistry protocols: Rink amide methylbenzhydrilamine (MBHA) resin (1 g, 0.66 mmol/g) was preswollen in *N*-methylpyrrolidone (NMP) for 2 h. Fmoc deprotection step was carried out with 20% piperidine in NMP (2 × 15 min), followed by washing with NMP (5 × 2 min) and DCM (2 × 2 min). Couplings of the building unit Fmoc-[β-*N*(Alloc)-ethyl]Gly OH to the resin and of Fmoc-amino-acid-OH (Fmoc-AA-OH) to the building unit were carried out according to a modified procedure published by Falb et al.[28] Briefly, FmocAAOH or building unit (3 equiv, 1.98 mmol) and bis-(trichloromethyl) carbonate (BTC, triphosgene) (1 equiv, 0.66 mmol) were suspended in DCM. 2,4,6-Collidine (10 equiv, 6.6 mmol) was added to the precooled suspension in an ice bath. After all the solids were dissolved (about 1 min), the solution was poured onto the resin and shaken for 3 h at room temperature. This coupling cycle was repeated once more. At the end of the second coupling cycle, the peptidyl-resin was washed with DCM (5 × 2 min). Capping was carried out after the anchoring of the building unit to the resin and was repeated twice by reaction of the peptidyl-resin with a mixture of acetic anhydride (1.1 mL, 0.5 M), diisopropyl amine (DIEA; 0.5 mL, 0.125 M), and *N*-hydroxybenzotriazole (HOBT; 0.05 g, 0.015 M) in dimethyl formamide (DMF, 25 mL). Capping was followed by resin wash with DMF (5 × 2 min), DCM (2 × 2 min), and NMP (2 × 2 min). Coupling of Fmoc-Trp(BOC)-OH to the building unit was carried out as follows: the Fmoc protecting group was removed from the peptidyl-resin with 20% piperidine in NMP (2 × 30 min) and the resin was washed (NMP, 5 × 2 min; DCM 5 × 2 min). Fmoc-Trp(BOC)-OH (3 equiv, 1.98 mmol) and BTC (1 equiv, 0.66 mmol) were suspended in dicholoromethane (DCM) and cooled in an ice bath for 30 min. 2,4,6-Collidine (10 equiv, 6.6 mmol) was added to the precooled suspension in an ice bath and mixed to received clear solution (1 min). Then the solution was added to the peptidyl-resin and the vessel was shaken for 3 h at RT. Washing steps were carried out with DCM (5 × 2 min). The cycle of Fmoc removal and coupling was followed with Fmoc-Arg(Pbf)-OH, Fmoc-DPhe-OH, and Fmoc-Phe-OH. All coupling steps were carried out using

BTC as the coupling agent in the same way that clarified above. The last amino acid on the peptidyl-resin (Phe) was acylated with 10 equiv of succinic anhydride in NMP, for 2 h at room temperature, in the presence of 1 equiv DMAP. The resin was washed with NMP (2 × 5 min) and DCM (2 × 5 min) and dried overnight in a desiccator. Alloc protecting group was removed with tetrakis (triphenylphosphine)Pd(0) (0.1 equiv, 0.066 mmol) in DCM containing acetic acid (5%) and *N*-methyl morpholin (2.5%) under argon. This step was carried out for 4 h with vigorous shaking in the dark. Washing steps were carried out with chloroform (8 × 2 min) and NMP with 0.5% DIEA (3 × 2 min). The peptide was cyclized by the addition of 6 equiv of PyBoP and 12 equiv of DIEA in NMP for 24 h (repeated twice) and then with 3 equiv of PyBoP and 6 equiv of DIEA in NMP. Washing steps were carried out with NMP (5 × 2 min) and DCM (5 × 2 min). The peptidyl-resin was dried under vacuum overnight.

Cleavage from the resin and removal of side chain protecting groups were carried out simultaneously using a precooled mixture of 95% TFA, 2.5% TDW, and 2.5% triisopropylsilane (TIS). After the resin was added, the mixture was agitated for 30 min in an ice bath, and then was shaken for 2.5 h at RT. The combined TFA filtrates were evaporated to dryness by a stream of nitrogen. The oily residue was triturated three times with cold ether to remove the scavengers, and the ether was removed by centrifugation. The dry crude peptide was dissolved in ACN/H_2O (1:1) and lyophilized. After final purification on preparative HPLC, product was obtained as white solid in 92% purity and 35% yield MS *m*/*z* 836.4 MH^+. Additional HPLC preparative purification yielded a compound with a purity of 98.5%, as assessed by NMR analysis.

NMR. Peptide **1** (see structure in Figure 3) was dissolved in 20% TFE-d_3, pH 6.25 (5.5 mM **1**, 20% TFE-d_3, 80% H_2O, 0.02% wt. NaN_3). Spectra were recorded at 303.0 K on a Bruker Avance 600 MHz DMX spectrometer operating at the proton frequency of 600.13 MHz using a 5 mm selective probe equipped with a self-shielded *xyz*-gradient coil. To characterize the sample, TOCSY,[29] COSY,[30] and ROESY[31,32] experiments were acquired under identical conditions. TOCSY spectra were recorded using the MLEV-17[29] pulse scheme for the spin lock during mixing periods of 150 ms and the residual water resonance was suppressed using the Watergate sequence.[33] ROESY was used instead of NOESY because the latter gave a null signal for this sample at this field. Water suppression was achieved by continuous wave saturation. 2D spectra were acquired and processed using the XWINNMR program (Bruker Analytische Messtechnik GmbH) and the SPARKY[34] software was used for analysis. Chemical exchange among conformers was confirmed by exchange peaks in the ROESY spectra, which show inverted signs compared to the ROE signals.[35] Assignment was done according to the sequential assignment methodology described by Wüthrich.[36]

Biochemistry. Preparation of Brush Border Membrane Vesicles. Brush border membrane vesicles (BBMVs) were prepared from combined duodenum, jejunum, and upper ileum by a Ca^{2+} precipitation method.[37–39] The intestines of five male Wistar rats, 200–250 gr, were rinsed with ice cold 0.9% NaCl and freed of mucus; the mucosa was scraped off the luminal surface with glass slides and put immediately into buffer containing 50 nM KCl and 10 mM Tris-HCl (pH 7.5, 4 °C) and then homogenated (Polytron PT 1200, Kinematica AG, Switzerland). $CaCl_2$ was added to a final concentration of 10 mM. The homogenate was left shaking for 30 min at 4 °C and then centrifuged at 10000 *g* for 10 min. The supernatant was then centrifuged at 48000 *g* for 30 min, and an additional two purification steps were undertaken by suspending the pellet in 300 mM mannitol and 10 mM Hepes/Tris (pH 7.5) and centrifuging at 24000 *g*/hr. Purification of brush border membranes was assayed using the brush border membrane enzyme markers γ-glutamyl transpeptidase (GGT), leucine amino peptidase (LAP), and alkaline phosphatase. During the course of these studies, enrichment in brush border membrane enzymes varied between 13- and 18-fold.

The tested molecule was mixed with purified BBMVs and incubated in 37 °C for 90 min. Duplicate samples were taken at time 0 and after 15, 30, 45, 60, and 90 min. The samples were diluted 1:1 with ice-cold acetonitrile, centrifuged (7500 g, 10 min, 4 °C), and sent to HPLC analysis.

Receptor Binding Assays. The receptor binding assays were performed by Cerep (France). Generally, transfected CHO cells were washed with binding buffer and distributed into 96-well plates. The cells were incubated for 2 h at 37 °C with 0.05 mL binding buffer in each well, containing a constant concentration of [^{125}I] NDP-α-MSH, and appropriate concentrations of an unlabeled ligand. After incubation, the cells were washed with ice-cold binding buffer and detached from the plates with 0.1 N NaOH. Radioactivity is counted and the data were analyzed with a software package for radio-ligand binding analyses by fitting it to formulas derived from the law of mass-action by the method generally referred to as computer modeling. The binding assays were performed in duplicate wells.

Functional Bioassay. HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μg of CRE/β-galactosidase reporter gene, as previously described.[8] Briefly, 5000–15000 post-transfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. A total of 48 h post-transfection, the cells were stimulated with 100 μL of peptide (10^{-4}−10^{-12} M) or forskolin (10^{-4} M) control in assay medium (DMEM containing 0.1 mg/mL BSA and 0.1 mM isobutylmethylxanthine) for 6 h. The assay media was aspirated and 50 μL of lysis buffer (250 mM Tris-HCl, pH 8.0, and 0.1% Triton X-100) was added. The plates were stored at −80 °C overnight. The plates containing the cell lysates were thawed the following day. Aliquots of 10 μL were taken from each well and transferred to another 96-well plate for relative protein determination. To the cell lysate plates, 40 μL of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μL of substrate buffer (60 mM sodium phosphate, 1 mM $MgCl_2$, 10 mM KCl, 5 mM β-mercaptoethanol, and 200 mg ONPG) was added to each well, and the plates were incubated at 37 °C. The sample absorbance, OD 405 nm, was measured using a 96-well plate reader (Molecular Devices). The relative protein was determined by adding 200 μL of 1:5 dilution BioRad G250 protein dye/water to the 10 μL cell lysate sample taken previously, and the OD 595 nm was measured on a 96-well plate reader (Molecular Devices). Data points were normalized both to the relative protein content and to the nonreceptor-dependent forskolin stimulation. The pA2 ($(K_i) - \log$ pA2) values were generated using the Schild analysis method.[40]

In Vitro Permeability Study. Growth and Maintenance of Cells. Caco-2 cells were obtained from ATCC, (Manassas, VA, U.S.A.) and then grown in 75 cm^2 flasks with approximately 0.5 × 10^6 cells/flask at 37 °C in 5% CO_2 atmosphere and at a relative humidity of 95%. The culture growth medium consisted of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% nonessential amino acids, and 2 mM L-glutamine. The medium was replaced twice weekly. All medium supplements were purchased from Biological Industries, Beth-Haemek, Israel.

Preparation of Cells for Transport Studies. For transport studies, cells in a passage range of 60–66 were seeded at a density of 25 × 10^5 cells/cm^2 on untreated culture inserts of polycarbonate membrane with 0.4 μm pores and surface area of 1 cm^2. The culture inserts containing Caco-2 monolayers were placed in 24 transwell plates, 12 mm, Costar. The culture medium was changed every other day. Transport studies were performed 21–23 days after seeding, when the cells were fully differentiated and the TEER (trans epithelial electrical resistance) values were stable (300–500 Ωcm^2).

Experimental Protocol. Transport study was initiated by medium removal from both sides of the monolayer and replacement with apical buffer (550 μL) and basolateral buffer (1200 μL), both warmed to 37 °C. The cells were incubated for a 30 min period at 37 °C with shaking (100 cycles/min). After the incubation period, the buffers were removed and replaced with 1200 μL of basolateral buffer at the basolateral side. Test solutions were warmed previously to 37 °C and added (600 μL) to the apical side of the monolayer.

Samples (50 μL) were taken from the apical side immediately at the beginning of the experiment, resulting in a 550 μL apical volume during the experiment. For the duration of the experiment, the cells were kept at 37 °C with shaking. At predetermined times (30, 60, 90, 120, 150, and 180 min), 200 μL samples were taken from the basolateral side and replaced with the same volume of fresh basolateral buffer to maintain a constant volume.

Data Analysis. The permeability coefficient (P_{app}) for each compound was calculated from the linear plot of drug accumulated versus time, using the following equation: $P_{app} = dQ/dt / (C_0 \times A)$, where dQ/dt is the steady state rate of the appearance of the drug on the receiver side, C_0 is the initial concentration of the drug on the donor side, and A is the surface area, 1.1 cm^2.

In Vivo Studies. All surgical and experimental procedures were reviewed and approved by the Animal Experimentation Ethics Committee of the Hebrew University Hadassah Medical Center, Jerusalem.

Pharmacokinetic Study. Studies were performed in conscious wistar male rats using jugular vein. An indwelling cannula was implanted 24 h before the pharmacokinetic experiment to allow full recovery of the animals from the surgical procedure. Animals ($n = 5$) received an iv bolus dose of 1 mg/kg of peptide **1** or 10 mg/kg for po administration ($n = 2$) dissolved in water. Blood samples (with heparin, 15 U/ml) were collected at several time points up to 24 h after peptide **1** administration. Plasma was separated by centrifugation (4000 *g*, 5 min, 4 °C) and stored at −70 °C pending analysis. Noncompartmental pharmacokinetic analysis was performed using *WinNonlin* software, standard edition version 5.0.1 (Scientific Consulting, Inc., Cary, NC).

Pharmacodynamic Study. ICR:Hsd (CD-1) male mice, 7–8 wks old were maintained in separate cages at 23 ± 1 °C on a 12 h light, 12 h dark cycle (0700–1900 h light). Mice were allowed ad libitum access to water and standard chow pellets. Upon arrival, mice were allowed to acclimate for 1 week. Following fasting for 16 h, the animals ($n = 24$) were subjected to a single oral gavage (po, 5 mL/kg) of peptide **1** (100 μg/ml) or vehicle (water). Immediately after administration, fixed food doses were added and reweighed after 1, 2, 3, 4, and 5 h. For chronic administration of peptide **1**, animals (3–4 wk) were weighed daily and then subjected to oral gavage (po, 5 mL/kg) of **1** (100 μg/ml) or vehicle (water).

Bioanalysis of Peptide 1. Brain Samples. The initial extraction of peptide **1** from the tissue was by single protein precipitation step using strong extraction reagent (A): 90% acetonitrile/10% formic acid and 50 mM ammonium formate thereafter; further purification of the analyte fraction was undertaken by adding another extraction step using reagent (A) diluted 2-fold with water. Peptide **2** was added to the brain samples as an internal standard (IS) prior to the extraction. Following the second extraction step, peptides **1** and **2** were analyzed successfully. Analysis of peptide **1** in rat brain using the API-3200 LC-MS/MS was developed and shown to be linear, accurate, reproducible, specific, and with high extraction recovery from rat brain.

Plasma/Buffer Samples. This study was performed using a HPLC-MS Waters Millenium instrument equipped with Micromass ZQ detector, Waters 600 Controller gradient pump, and Waters 717 auto sampler. Nitrogen flow was 500 L/hr; source temperature was 400 °C; the cone voltage was 60 V; the column was Xterra MS C_{18} 2.1 × 150 mm (Waters). The mobile phase at 0.3 mL/min was 30% acetonitrile, 0.1% formic acid, and 0.05% TFA, with a linearity range of 0.025–1 μg/mL.

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