Published in final edited form as:

Biopolymers. 2008 ; 90(5): 671–682. doi:10.1002/bip.21057.

Structure-Activity Relationship and Metabolic Stability Studies of Backbone Cyclization and N-Methylation of Melanocortin Peptides

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Abstract

Backbone cyclization (BC) and N-methylation have been shown to enhance the activity and/or selectivity of biologically active peptides and improve metabolic stability and intestinal permeability. In this study, we describe the synthesis, structure-activity relationship (SAR) and intestinal metabolic stability of a backbone cyclic peptide library, BL3020, based on the linear α -Melanocyte stimulating hormone analog Phe-D-Phe-Arg-Trp-Gly. The drug lead, BL3020-1, selected from the BL3020 library (compound 1) has been shown to inhibit weight gain in mice following oral administration. Another member of the BL3020 library, BL3020-17, showed improved biological activity towards the mMC4R, in comparison to BL3020-1, although neither were selective for MC4R or MC5R. N-methylation, which restrains conformational freedom while increasing metabolic stability beyond that which is imparted by BC, was used to find analogs with increased selectivity. N-methylated backbone cyclic libraries were synthesized based on the BL3020 library. SAR studies showed that all the N-methylated backbone cyclic peptides demonstrated reduced biological activity and selectivity for all the analyzed receptors. N-methylation of active backbone cyclic peptides destabilized the active conformation or stabilized an inactive conformation, rendering the peptides biologically inactive. N-methylation of backbone cyclic peptides maintained stability to degradation by intestinal enzymes.

Keywords

backbone cyclization; N-methylation; Fmoc chemistry; structure activity relationship

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INTRODUCTION

It is well recognized that peptides generally lack single receptor selectivity¹ and have low bioavailability profiles due to their fast rate of metabolism. Many approaches have been developed to overcome these limitations, among which are backbone cyclization (BC)² and N-methylation.³⁻⁸ These approaches are general methods for conformationally constraining peptides.^{9,10} BC and cycloscan are general methods by which conformational constraints can be imposed on peptides. BC is formed by covalently connecting atoms in the backbone of a target linear peptide via a linker to form a ring. BC has been shown to dramatically enhance the metabolic stability of peptides in serum and intestinal enzymes.¹¹ A library of backbone cyclic peptides has a basic parent peptide sequence with the same side chain pharmacophores and hence the same potential bioactivity,^{2,10} but they differ in their ring size and chemistry, conferring conformational diversity. Discrete conformational perturbations, introduced by changing the linker properties, create cyclic peptides that sample the conformational space of the parent peptide. The advantage of BC over other methods of peptide cyclization is that cyclization is performed mainly through backbone atoms without using side chains that are essential for biological activity. This method has been shown to improve the pharmacological selectivity of a given peptide, as demonstrated for substance P⁸ and somatostatin¹² analogs.

N-methylated peptides were shown to have improved pharmacological properties such as lipophilicity,¹³ proteolytic stability,^{13,14} bioavailability,¹⁵⁻¹⁷ and conformational rigidity.¹⁸ They have also shown enhanced potency¹⁹⁻²² and new receptor subtype selectivity.²³⁻²⁵ For example, N-methylated cyclic RGD peptides²² and dermorphin analogs²⁶ have been shown to be more potent following mono N-methylation. Multiple N-Methylation (MNM) is an approach where a library of all possible N-methylated peptide analogs, based on the sequence of a linear or cyclic bioactive peptide, is synthesized and screened to select an active N-methylated analog.¹⁷ This approach has led to the discovery of a new selective, metabolically stable, intestinally permeable somatostatin analog,¹⁷ and RGD analogs with new receptor selectivity.⁶ N-methylation and the MNM approach are recognized as useful methods for enhancing selectivity and improving pharmacological properties of peptides.

α -Melanocyte stimulating hormone (α MSH) is part of the melanocortin pathway, which controls the metabolic balance in the body and regulates it.²⁷ This 13-amino acid hormone can bind to five different melanocortin receptors (MC1R-MC5R) which belong to the super family of seven transmembrane-spanning G-protein-coupled receptors. The active site of α MSH possess the same common structural motif, His-Phe-Arg-Trp, for all the five receptors.²⁸⁻³¹ It is important to note that α MSH binding to each receptor stimulates different physiological pathways in the body. The MC1R is involved in pigmentation and animal coat coloration.^{32, 33} The MC2R regulates lipogenesis, glucocorticoid, and aldosterone production.³⁴ The MC3R and MC4R have been identified in knockout mice to be involved in feeding behavior, obesity, metabolism, and energy homeostasis.³⁵ MC5R knockout mice showed the role of this receptor in exocrine gland function.³⁶

Backbone cyclic peptides based on the sequence Phe⁶-D-Phe-Arg-Trp⁻Gly¹⁰ have been subjected to extensive structure-activity relationship (SAR) studies.¹¹ This sequence is an analog of the minimal α MSH sequence that activates the MC4 receptor.³¹ BL3020-1 (compound 1, Figure 1A) from this library was found to have high intestinal permeability, high stability to intestinal enzymes, and found to have bound and activated the MC4R. Oral administration of compound 1 in mice showed reduced food intake (~40% in 24 h) and inhibition in body weight gain (over 12 days) in comparison to the control group.^{11,37} EC₅₀ analysis showed that compound 1 binds and activates the MC4 and MC5 receptors with the same selectivity (EC₅₀ 4.0 $\pm$ 0.6 nM and 7.0 $\pm$ 0.4 nM, respectively).^{11,37}

Two libraries were synthesized, comprising 37 backbone cyclic (BL3020) and N-methylated backbone cyclic (N-Me) peptides with the same sequence as compound 1 (Phe⁶-D-Phe-Arg-Trp⁻Gly¹⁰-NH₂) and a lactam bridge between the N terminus and the backbone nitrogen of the C-terminal Gly (see Figure 1). The peptides in the BL3020 library differed in ring size and ring chemistry, whereas the peptides in the N-Me libraries differed also in the number and position of the N-Me group. These libraries were screened to select peptide(s) with improved selectivity and activity towards MC4R, while retaining resistance against intestinal enzymes. Such peptide(s) would have the potential to serve as improved antiobesity drug leads, while avoiding adverse effects caused by activation of other MCR subtypes. Nuclear magnetic resonance (NMR)-derived structures of compound 1 were compared with the homology model of α MSH in MC4R,³⁸ which showed a similar orientation of Arg⁸-Trp⁹ but not D-Phe⁶ and Phe⁷ (Safrai et al., unpublished results), suggesting that the bridge size and N-methylation of D-Phe⁶ and Phe⁷ may effect the activity and selectivity. SAR studies were performed by activating mouse melanocortin receptors mMC1R, mMC3R, mMC4R, and mMC5R, and screening for metabolic stability was done using the brush border membrane vesicles (BBMV)³⁶ *in vitro* model. Peptide BL3020-17 from the BL library showed increased activity towards MC4R, but N-methylation decreased biological activity and selectivity for all the analyzed receptors while maintaining resistance towards enzymatic degradation.

MATERIALS AND METHODS

Chemistry General

Protected amino acids, 9-fluorenylmethyloxycarbonyl-*N*-hydroxysuccinimide (Fmoc-OSu), bromo-tris-pyrrolidone-phosphonium hexafluorophosphate (PyBrop), Rink amide methylbenzhydrylamine (MBHA) polystyrene resins, and organic reagents 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), trifluoroacetic acid (TFA) and solvents for high performance liquid chromatography (HPLC) were purchased from Bio-Lab (Jerusalem, Israel). Glyoxylic acid, 1,2-diaminoethane, 1,3-diaminopentane, and 1,4-diaminobutane were purchased from Merck (Darmstadt, Germany); tetrakis (triphenylphosphine)palladium (0) was purchased from ACROS (Geel, Belgium). Organic solvents were purchased from Frutarom (Haifa, Israel). NMR spectra 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 were 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 dropped 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) (L-6200A pump, Merck-Hitachi, Japan), using a Vydac preparative RP column (C8, 22 × 250 mm, catalog number 218TP1022). 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.

Solid Phase Peptide Synthesis of N-Methylation Libraries

The synthesis was performed^{3,11} in a reaction vessel equipped with a sintered glass bottom, following general Fmoc chemistry protocols: Rink amide methylbenzhydrylamine (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 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.³⁹ (3 equiv., 1.98 mmol) and bis-(trichloromethyl) carbonate (BTC, triphosgene) (1 equiv., 0.66 mmol) were suspended in DCM. 2,4,6-trimethylcollidine (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 anchoring the building unit to the resin and was repeated twice by reacting the peptidyl-resin with a mixture of acetic anhydride (1.1 mL, 0.5M), diisopropylethyl amine (DIEA) (0.5 mL, 0.125M), and *N*-hydroxybenzotriazole (HOBT) (0.05 g, 0.015M) in dimethyl formamide (DMF) (25 mL). Capping was followed with a 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 piperidine in NMP (20%, 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 dichloromethane and cooled in an ice bath for 30 min. 2,4,6-collidine (10 equiv., 6.6 mmol) was added, and after all the solids were dissolved (1 min), the solution was added to the peptidyl-resin and the vessel was shaken for 3 h at room temperature. Washing steps were carried out with DCM (5×2 min). The cycle of Fmoc removal and coupling was followed with Fmoc-DPhe-OH and Fmoc-Phe-OH. All the coupling steps were carried out using BTC as the coupling agent as described earlier. 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), dried overnight in a desiccator, and removal of the Alloc protecting group from the building unit was performed 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). After Alloc deprotection, the peptide was cyclized by adding 6 equiv. PyBoP and 12 equiv. DIEA in NMP for 24 h (repeated twice) and then with 3 equiv. PyBoP and 6 equiv. 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 over night. 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 room temperature. 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/H₂O (1:1) and lyophilized.

Functional Bioassay

HEK-293 cells stably expressing the melanocortin receptors were transfected with 4 μ g of CRE/ β -galactosidase reporter gene, as previously described.²⁹ Briefly, 5000–15,000 post-transfection cells were plated into 96-well Primera plates (Falcon) and incubated overnight. At 48 h post-transfection, the cells were stimulated with 100 μ L of peptide (10^{-4} to 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 μ L 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 μ L were taken from each well and transferred to another 96-well plate for relative protein

determination. To the cell lysate plates, 40 μ L of phosphate-buffered saline with 0.5% BSA was added to each well. Subsequently, 150 μ L substrate buffer (60 mM sodium phosphate, 1 mM MgCl₂, 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 μ L of 1:5 dilution BioRad G250 protein dye/water to the 10 μ L 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.

Preparation of Brush Border Membrane

Vesicles—BBMVs were prepared from combined duodenum, jejunum, and upper ileum by a Ca²⁺ precipitation method.^{40–42} The intestines of five male Wistar rats, 200–250 g, were rinsed with ice cold 0.9% NaCl solution and freed of mucus; the mucosa was scraped off the luminal surface with glass slides and put immediately into buffer containing 50 mM KCl and 10 mM Tris-HCl (pH 7.5, 4°C) and then homogenated (Polytron PT 1200, Kinematica AG, Switzerland). CaCl₂ was added to a final concentration of 10 mM. The homogenate was left shaking for 30 min at 4°C and then centrifuged at 10,000g for 10 min. The supernatant was then centrifuged at 48,000g 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 24,000g/h. 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 ACN, centrifuged (7500g, 10 min, 4°C), and sent to HPLC analysis.

RESULTS

Backbone cyclic and N-methylated backbone cyclic pentapeptides were synthesized and their biological activities towards mouse MC1R, MC3R, MC4R, and MC5R were determined.

Chemical Synthesis and Characterization

Two libraries of backbone cyclic and N-methylated backbone cyclic pentapeptides were synthesized, designated BL3020 and N-Me. The latter was further divided into three sub-libraries, N-Me-1, N-Me-2, and N-Me-3 according to the position of N-methylation. All BL3020 peptides contain the parent sequence Phe⁶-D-Phe-Arg-Trp-Gly¹⁰-NH₂ in which a lactam bridge connects the N-terminus to the N ^{α} of the C-terminal Gly, resembling the ring position of the model compound 1^{11,37} (Figure 1A and Table I). All the peptides differ in the length of the alkyl chains, the position of the lactam bridge in the ring and ring chemistry. The position of the N-methylation in the N-Me-1 sub-library was *N*-methyl Phe⁶ (Figure 1B and Table II); All the peptides in the N-Me-2 sub-library, had both *N*-methyl Phe⁶ and *N*-methyl D-Phe⁷ (Figure 1C and Table III); and the N-Me-3 sub-library was precyclic analogs of peptides from the N-Me-2 sub-library (Figure 1D and Table IV). All the peptides were synthesized on Rink amide MBHA resin by the Fmoc chemistry as described by Hess et al.¹¹ N-methylation was performed on resin by the method described by Biron et al.³ The cyclization was performed on-resin after removing the Alloc protecting group from the N ^{α} (ω -aminoalkyl)Gly building unit. The peptides of the N-Me-3 sub-library were the same peptides as those of the N-Me-2 sub-library, except that the cyclization step was not performed in the N-Me-3 library, and the peptides were removed from the resin after Alloc deprotection (see Figure 1). All peptides were purified to homogeneity using preparative RP-HPLC. The

purity and characterization of these peptides was assessed by both mass spectrometry and analytical RP-HPLC.

Biological Evaluation

Tables VI-VIII summarize the biological activity at the mouse melanocortin receptors, mMC1R, mMC3R, mMC4R, and mMC5R of the 37 peptides. Previously, we have demonstrated that compound 1 selectively activates the MC4R and the MC5R in the low nanomolar range ($EC_{50} = 4.0$ and 7.0 nM, respectively) which indicates agonist^{1/4} activity, whereas the control peptides NDP-MSH showed very high affinity agonist activity towards all the analyzed receptors with no selectivity.¹¹ Figure 2 summarizes the EC_{50} values of a sample of BL3020 peptides towards mMC4R. Figures 3A-3C summarizes the EC_{50} values of a sample of peptides from each of the three N-methylation sub-libraries towards mMC4R. Figure 3D demonstrates the EC_{50} values of all N-Me-1 peptides towards mMC3R.

Mouse Melanocortin-1 Receptor—The peripheral skin melanocortin receptor, MC1R, is involved in human skin pigmentation^{32,43} and animal coat coloration.³³ Compound 1 has been shown to possess low stimulatory activity at the mMC1R ($EC_{50} = 64$ nM).¹¹ The results of the current study showed that the stimulatory activity at the mMC1R for BL3020 peptides was between 28 nM for BL3020-17 to 3.3 μ M for BL3020-11. Thus, the biological activity at the mMC1R of BL3020-17 was increased by 2.2 fold, while that of BL3020-11 was decreased by 51 fold, in comparison to compound 1 (Table V). The biological activity of the N-Me-1 peptides was between 720 nM for N-Me-1-1 to 12.68 μ M for N-Me-1-4, which is 11- to 198-fold decreased potency, respectively, in comparison to compound 1 (Table VI). The N-Me-2 peptides showed stimulatory activity at the mMC1R between 1.05 μ M for N-Me-2-5 to 8.67 μ M for N-Me-2-8, which is 15.6- to 135-fold decreased potency, respectively, in comparison to compound 1 (Table VII). N-Me-3 peptides stimulated the mMC1R at 1.35 μ M for N-Me-3-1 to 36.7 μ M for N-Me-3-11, which is a 21- to 573-fold decrease in potency, respectively, in comparison to compound 1 (Table VIII). These results showed that the stimulatory activity at the mMC1R for all analyzed peptides was reduced in comparison to that found for compound 1, except for two peptides, BL3020-8 and BL3020-17 (Table V). Specifically, BL3020-17 showed the highest biological activity at the mMC1R, which was 2.2-fold more than that of compound 1.

Mouse Melanocortin-3 Receptor—The MC3R is expressed both peripherally and centrally and appears to be involved in metabolism and energy homeostasis.^{43,44} Compound 1 has been shown to have an EC_{50} value of 770 nM at the mMC3R.¹¹ The results of the current study showed that the stimulatory activity at the mMC3R for BL3020 peptides was between 33.0 nM for BL3020-14 to over 100 μ M for BL3020-6 and BL3020-11. Thus, the biological activity at the mMC3R of BL3020-14 was increased by 23.3-fold, whereas those of BL3020-6 and BL3020-11 were fully depressed in comparison to compound 1 (Table V). N-methylation libraries results showed that all the peptides were devoid of any potency at the mMC3R (Tables VII-9 and Figure 3D), except for N-Me-2-11. This peptide stimulated the mMC3R at 10.38 μ M, which is 13.5-fold decreased potency in comparison to compound 1 (Table VII). The N-Me-2 library differed from the other two libraries in N-methylamino acids on Phe⁶ and D-Phe⁷. All the peptides showed less potency to MC3R, in comparison to compound 1, except for BL3020-14. It is interesting to note that all the peptides that had EC_{50} values above 100 μ M (Tables VII-9 and Figure 3D) had no agonist activity, which indicates a potential role as antagonists at the mMC3R.

Mouse Melanocortin-4 Receptor—The central MC4R has been identified as physiologically participating in food consumption⁴⁵ and obesity in mice⁴⁶ with several polymorphisms of the MC4R observed in obese humans.⁴⁷⁻⁴⁹ Compound 1 was shown to have

an EC₅₀ value of 4.0 nM at the mMC4R.¹¹ BL3020–17 (Table I) stimulated the mMC4R with an EC₅₀ of 1.8 nM, a 2.2-fold increase in potency compared to that found for compound 1, whereas BL3020–10 stimulated the mMC4R with an EC₅₀ of 3.7 μM, a 925-fold decrease potency in comparison to compound 1 (Figure 2 and Table V). Among the N-Me-1 peptides, N-Me-1–3 stimulated the mMC4R with an EC₅₀ of 96.0 nM, which is 24-fold decreased potency when compared with that found for compound 1 (Figure 3A and Table VI). N-Me-1–4 stimulated the mMC4R with an EC₅₀ of 1.91 μM, which is 478-fold decreased potency when compared with compound 1 (Figure 3A and Table VI). The other peptides of this library showed EC₅₀ values within this range (Figure 3A and Table VI). N-Me-2 peptides (N-Me-2–1 to N-Me-2–18) showed stimulatory activity at the mMC4R between 498 μM for N-Me-2–14 to 4.9 μM for N-Me-2–1 and N-Me-2–12, which is 124- to 1225-fold decreased potency, respectively, in comparison to compound 1 (Figure 3B, Table VII). N-Me-3 peptides stimulated the mMC4R at 336 nM for N-Me-3–16 to 1.79 μM for N-Me-3–4, which is 84- to 447-fold decreased potency, respectively, in comparison to compound 1 (Figure 3C, Table VIII). The stimulatory activity at the mMC4R for all analyzed peptides was reduced in comparison to that found for compound 1, except for BL3020–17. However, although this peptide showed higher biological activity at the mMC4R when compared with compound 1, it was not selective for this receptor.

Mouse Melanocortin-5 Receptor—The peripheral MC5R is expressed in a variety of tissues and has been implicated as physiologically participating in the role of exocrine gland function.^{34,36,50} The agonist activity of compound 1 at the mMC5R was shown to have an EC₅₀ of 7.0 nM.¹¹ The results of the current study showed that the stimulatory activity at the mMC5R for BL3020 peptides was between 2 nM for BL3020–8 to 390 nM for BL3020–16 (Table I). The biological activity at the mMC5R of BL3020–8 was increased by 3.5-fold, while that of BL3020–16 was decreased 56-fold, in comparison to compound 1 (Table V). The N-Me-1 peptides were between 69.0 nM for N-Me-1–3 to 1.61 μM for N-Me-1–6, which is 9.8- to 230-fold decreased potency, respectively, in comparison to compound 1 (Table VI). N-Me-2 peptides showed stimulatory activity at the mMC5R between 189 μM for N-Me-2–9 to 8.0 μM for N-Me-2–5, which is 27- to 1141-fold decreased potency, respectively, in comparison to compound 1 (Table VII). N-Me-3 peptides stimulated the mMC5R at 290 nM for N-Me-3–16 to 849 nM for N-Me-3–10, which is 21- to 121-fold decreased potency, respectively, in comparison to compound 1 (Table VIII). The stimulatory activity at the mMC5R for all analyzed peptides was reduced in comparison to that found for compound 1, except for BL3020–8 and BL3020–17.

Metabolic Stability

Metabolic stability was studied with BBMV.³⁶ The stability of the N-methylated analogs N-Me-1–3 and N-Me-2–8 was compared to that of compound 1 and the precyclic analog N-Me-3–4. The cyclic N-methylated analogs had a 100% recovery after 90 min, and the precyclic N-Me-3–4 was found to be less stable to intestinal enzymes, with a 30% reduced concentration after 90 min (see Figure 4).

DISCUSSION

Backbone cyclic peptide library (BL3020), and N-methylated (N-Me) libraries, were synthesized and their activity determined. This is the first case in which BC and the MNM approaches were combined. Previously, the MNM approach has been applied to end-to-end cyclic peptides.^{6,17} In such MNM library, the diversity is only in the position and number of the N-Me groups. In the BC-MNM approach, there are two types of libraries: Type I stems from a previously screened BC library where an active peptide is selected and used for the MNM library. In this case, the diversity is the same as in the end-to-end MNM cyclic peptide

library. In the second library, type II, the diversities of both MNM and BC are combined, such that each N-methylated peptide is the basis of a library that has ring size and chemistry diversity. Obviously, the size of the library of type II is much bigger than the size of the library of type I. Here we describe the synthesis and SAR of the peptides in the BC-MNM library, based on the α MSH stem peptide, with type II diversity.

All the peptides in both the BC and the BC-MNM libraries had the same primary sequence: Phe-D-Phe-Arg-Trp-Gly-NH₂. They also had the same ring position. The N-Me library was further divided into three sub-libraries. Although peptides in the BL3020 library differed in ring size and chemistry, the peptides in the N-Me library differed in these two diversity parameters and also in their N-methylation. The N-Me-1 sub-library contains N-Me-Phe⁶; and the N-Me-2 sub-library contain both N-Me-Phe⁶ and N-Me-D-Phe⁷; and the N-Me-3 sub-library comprised precyclic peptides with the same modifications as the N-Me-2 peptides but not cyclic (see Figure 1).

The SAR studies demonstrated significant difference between the two main libraries, where several of the BL3020 peptides had enhanced biological activity towards to the different mMCRs when compared with compound 1, whereas all the peptides in the N-Me libraries had significantly reduced biological activity to relative to compound 1. BL3020-17 (with a ring size of 21) and BL3020-14 (with a ring size of 25) stimulated the mMCRs at lower concentrations than compound 1. These results indicate that the ring size has a significant effect on their biological activity on the mMCRs. The results of the peptides from the N-Me libraries showed that none of them stimulated mMCRs at concentrations below those of compound 1. N-Me-1-6, based on BL3020-17, and N-Me-2-17, based on BL3020-14, stimulated the mMC4R at significantly higher concentrations than compound 1. BL3020-17 and N-Me-1-6 have the same ring size and ring chemistry: The only difference between them is the N-methylated amino acid on Phe⁶, showing that N-methylation impaired the biological activity towards mMC4R and that the active conformation is probably destabilized by N-methylation at this position.

Ring size, ring chemistry, and N-methylation also dramatically effected receptor selectivity. It is important to note that the BL3020 peptides showed both mMCR4 and mMCR5 activity, indicating that similar bioactive conformations activate these receptors.

The lack of biological activity due to N-methylation is in contrast to findings of Biron et al.¹⁷ and Chatterjee and co-worker.⁴ Where N-methylation was shown to increase bioavailability and in several cases also the interaction of the drug with its targets, and hence its overall activity. Here, N-methylation reduced biological activity, probably by changing the active conformation and/or by increasing the rigidity of the molecule. These changes may prevent hydrogen bonding that stabilizes the peptide in the active conformation (Safrai et al., unpublished results) and thus can prevent the interaction with the receptor.

The backbone cyclic peptides (with or without N-methylation) maintain their metabolic stability: Our results demonstrate that N-methylation does not affect the intestinal stability of the backbone peptides. BC stabilization of the peptides was demonstrated as the N-methylation precyclic analog was found to be more susceptible to degradation by the intestinal enzymes than the cyclic analog.

CONCLUSIONS

The sequence Phe⁶-D-Phe-Arg-Trp-Gly¹⁰-NH₂ derived from α MSH was the basis of two libraries: (a) An amino end to N-backbone cyclic ring size and ring chemistry library (the BL3020 library), and (b) An extensive, N-methylated BC-MNM library based on the BC library. A previously discovered BC peptide (compound 1) that had MC4R and MC5R

selectivity and oral *in vivo* activity was used as a lead. Screening these libraries for activation of various melanocortin receptors revealed that some peptides in the BL3020 library have improved activity and selectivity, when compared with compound 1, whereas the peptides in the BC-MNM library had reduced activity. N-methylation at position 6 and 7 render the BC peptide inactive. Peptides of the BC-MNM maintained their intestinal metabolic stability when compared with their precyclic analogs.

Acknowledgments

This work is part of the Ph.D. dissertations of Yaniv Linde and Oded Ovadia.

Contract grant sponsor: US-Israeli Binational Science Foundation (BSF)

Contract grant number: 2005-193

Contract grant sponsor: NIH

Contract grant number: DK57080

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A Compound 1 **B N-Me-1 Library** **C N-Me-2 Library** **D N-Me-3 Library** **FIGURE 1.**

Structures of compound 1 and general structures of the “N-methylation” sub-libraries (A). The structure of compound 1 (B). General structures of N-Me-1 (C). General structures of N-Me-2 (D). General structures of pre-cyclic N-Me-3 libraries. The arrows indicate the N-methylation position.

FIGURE 2.

Illustration of the chosen BL3020 peptides possessing partial agonist pharmacology at the mouse MC4R (A). BL3020-6, 7, 10, 11, and 13 agonist pharmacology at the mouse MC4R (B). BL3020-4, 8, 14, 15, and 17 agonist pharmacology at the mouse MC4R. The peptides NDP and compound 1 are included as controls to illustrate the maximal response observed for full agonists using this assay. HEK-293 cell in 96-well plate stably expressing the melanocortin receptors were incubated with the tested molecules overnight. The sample absorbance, OD 405 nm, was measured using a 96-well plate reader (Molecular Devices).

FIGURE 3.

Illustration of the chosen N-methylation peptides possessing partial agonist pharmacology at the mouse MC3R and agonist pharmacology at the mouse MC4R. (A) N-Me-1 library agonist pharmacology at the mouse MC4R. (B) N-Me-2 library agonist pharmacology at the mouse MC4R. (C) N-Me-3 library agonist pharmacology at the mouse MC4R. (D). N-Me-2 library agonist pharmacology at the mouse MC3R. The peptides NDP and compound 1 are included as controls to illustrate the maximal response observed for full agonists using this assay. HEK-293 cell in 96-well plate stably expressing the melanocortin receptors were incubated with the tested molecules overnight. The sample absorbance, OD 405 nm, was measured using a 96-well plate reader (Molecular Devices).

FIGURE 4. Metabolic stability in brush border membrane vesicles (BBMV), (■) N-Me-3-4 (pre-cyclic), (▼) N-Me-1-3 (●) N-Me-2-8, and (◆) Compound 1.

Table I

Structure of the BL3020 Library

Peptide Name	Ring Size	<i>m</i>	<i>n</i>	Sequence
Compound 1 ^a	20	2	2	c(Phe(C2)-D-Phe-Arg-Trp-Gly(N2)NH ₂)
BL3020-4 ^a	21	3	2	c(Phe(C3)-D-Phe-Arg-Trp-Gly(N2)NH ₂)
BL3020-6	22	3	3	c(Phe(C3)-D-Phe-Arg-Trp-Gly(N3)NH ₂)
BL3020-7	24	5	3	c(Phe(C5)-D-Phe-Arg-Trp-Gly(N3)NH ₂)
BL3020-8 ^a	21	2	3	c(Phe(C2)-D-Phe-Arg-Trp-Gly(N3)NH ₂)
BL3020-9	23	5	2	c(Phe(C5)-D-Phe-Arg-Trp-Gly(N2)NH ₂)
BL3020-10	22	4	2	c(Phe(C4)-D-Phe-Arg-Trp-Gly(N2)NH ₂)
BL3020-11	22	2	4	c(Phe(C2)-D-Phe-Arg-Trp-Gly(N4)NH ₂)
BL3020-12	23	3	4	c(Phe(C3)-D-Phe-Arg-Trp-Gly(N4)NH ₂)
BL3020-13	24	4	4	c(Phe(C4)-D-Phe-Arg-Trp-Gly(N4)NH ₂)
BL3020-14	25	5	4	c(Phe(C5)-D-Phe-Arg-Trp-Gly(N4)NH ₂)
BL3020-15 ^a	23	4	3	c(Phe(C4)-D-Phe-Arg-Trp-Gly(N3)NH ₂)
BL3020-16 ^a	21	<i>a</i>	2	A
BL3020-17	21	<i>b</i>	2	B

^aThe dicarboxylic acyl spacer (*m*) was replaced by *o*-phthaloyl spacer. Hess et al.¹¹

^bThe dicarboxylic acyl spacer (*m*) was replaced by *dimethylglutaric acid* spacer. An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.0 mL/min. The peptide purity (>95%) was determined by HPLC at a wavelength of 220 nm. Gly(Nx) represents N α (ω -amino(alkyl)_x)Gly.

Table II

Structure of the N-Me-1 Sub-Library

Peptide Name	Ring Size	<i>m</i>	<i>n</i>	Origin
N-Me-1-1	20	2	2	BL3020-1
N-Me-1-2	21	3	2	BL3020-4
N-Me-1-3	21	<i>a</i>	2	BL3020-16
N-Me-1-4	22	4	2	BL3020-10
N-Me-1-6	21	<i>b</i>	2	BL3020-17

^aThe dicarboxylic acyl spacer (*m*) was replaced by *o*-phthaloyl spacer.

^bThe dicarboxylic acyl spacer (*m*) was replaced by *dimethylglutaric acid* spacer. An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.0 mL/min. The peptide purity (>95%) was determined by HPLC at a wavelength of 220 nm.

Table III

Structure of the N-Me-2 Sub-Library

Peptide Name	Ring Size	<i>m</i>	<i>n</i>	Origin
N-Me-2-1	20	2	2	BL3020-1
N-Me-2-2	21	3	2	BL3020-4
N-Me-2-4	22	4	2	BL3020-10
N-Me-2-5	23	5	2	BL3020-9
N-Me-2-6	21	<i>a</i>	2	BL3020-17
N-Me-2-7	21	2	3	BL3020-8
N-Me-2-8	22	3	3	BL3020-6
N-Me-2-9	22	<i>b</i>	3	BL3020-16 ^c
N-Me-2-10	22	4	3	BL3020-15
N-Me-2-11	23	5	3	BL3020-7
N-Me-2-12	23	<i>b</i>	3	BL3020-17 ^c
N-Me-2-13	22	2	4	BL3020-11
N-Me-2-14	23	3	4	BL3020-12
N-Me-2-15	23	<i>b</i>	4	BL3020-16 ^c
N-Me-2-16	24	4	4	BL3020-13
N-Me-2-17	25	5	4	BL3020-14
N-Me-2-18	26	<i>b</i>	4	BL3020-17 ^c

^aThe dicarboxylic acyl spacer (*m*) was replaced by *dimethylglutaric acid* spacer. An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.0 mL/min. The peptide purity (>95%) was determined by HPLC at a wavelength of 220 nm.

^bThe dicarboxylic acyl spacer (*m*) was replaced by *o-phthaloyl* spacer.

^cThere are differences from the structures at Table I on the ring size and N-methylation position.

Table IV

Structure of the Pre-Cyclic N-Me-3 Sub-Library

Peptide Name	Ring Size	<i>m</i>	<i>n</i>	Origin
N-Me-3-1	–	2	2	BL3020-1
N-Me-3-3	–	<i>a</i>	2	BL3020-16
N-Me-3-4	–	4	2	BL3020-10
N-Me-3-10	–	4	3	BL3020-15
N-Me-3-11	–	5	3	BL3020-7
N-Me-3-16	–	4	4	BL3020-13

^aThe dicarboxylic acyl spacer (*m*) was replaced by *o*-phthaloyl spacer. An analytical Vydac C18 column (Vydac 218TP104) was used with a flow rate of 1.0 mL/min. The peptide purity (>95%) was determined by HPLC at a wavelength of 220 nm.

Table V

Functional Activity of BL3020 Library at the Mouse Melanocortin Receptors

Ref ^a	mMC1R (nM)	mMC3R (nM)	mMC4R (nM)	mMC5R (nM)
NDP-MSH	0.0081 ± 0.001	0.063 ± 0.004	0.097 ± 0.016	0.078 ± 0.0084
Compound 1 ^a	64 ± 14	770 ± 175	4.0 ± 0.6	7.0 ± 0.4
BL3020-4 ^a	120 ± 28	1460 ± 145	18 ± 4	20 ± 4
BL3020-6	590 ± 170	>100,000	29 ± 3	51 ± 17
BL3020-7	1600 ± 700	12,000 ± 2600	6.4 ± 1.0	73 ± 13
BL3020-8 ^a	28 ± 12	1260 ± 385	4 ± 2	2 ± 1
BL3020-10	740 ± 120	11,500 ± 5200	3706 ± 4	92 ± 6
BL3020-11	3300 ± 2200	>100,000	35 ± 6	113 ± 15
BL3020-12	270 ± 84	5530 ± 300	17 ± 8	25 ± 1
BL3020-13	590 ± 77	7900 ± 1940	9.5 ± 2.0	50 ± 14
BL3020-14	340 ± 137	33 ± 17	9.0 ± 0.4	16 ± 4
BL3020-15 ^a	240 ± 41	7750 ± 1960	5.0 ± 0.1	26 ± 17
BL3020-16 ^a	550 ± 97	20,500 ± 2100	150 ± 22	390 ± 120
BL3020-17	28 ± 5	840 ± 150	1.8 ± 0.1	4 ± 1

^aHess et al.¹¹

Table VI

Functional Activity of N-Me-1 Library at the Mouse Melanocortin Receptors

Ref	mMC1R (nM)	mMC3R (nM)	mMC4R (nM)	mMC5R (nM)
NDP-MSH	0.0081 ± 0.001	0.063 ± 0.004	0.097 ± 0.016	0.078 ± 0.0084
Compound 1	64 ± 14	770 ± 175	4.0 ± 0.6	7.0 ± 0.40
N-Me-1-1	720 ± 397	>100,000	100 ± 47	200 ± 35
N-Me-1-2	743 ± 210	>100,000	175 ± 96	392 ± 89
N-Me-1-3	930 ± 156	>100,000	96 ± 46	69 ± 13
N-Me-1-4	12,680 ± 9150	>100,000	1913 ± 878	820 ± 132
N-Me-1-6	1490 ± 367	>100,000	291 ± 127	1610 ± 727

Table VII

Functional Activity of N-Me-2 Library at the Mouse Melanocortin Receptors

Ref	mMC1R (nM)	mMC3R (nM)	mMC4R (nM)	mMC5R (nM)
NDP-MSH	0.0081 ± 0.001	0.063 ± 0.004	0.097 ± 0.016	0.078 ± 0.0084
Compound 1	64 ± 14	770 ± 175	4.0 ± 0.6	7.0 ± 0.40
N-Me-2-1	2510 ± 610	>100,000	4900 ± 1800	4540 ± 1400
N-Me-2-2	4100 ± 190	>100,000	2500 ± 330	1430 ± 565
N-Me-2-4	1196 ± 670	>100,000	2877 ± 659	2960 ± 995
N-Me-2-5	1049 ± 312	>100,000	2416 ± 498	7990 ± 460
N-Me-2-6	4900 ± 1030	>100,000	4350 ± 385	7010 ± 2900
N-Me-2-7	8220 ± 7500	>100,000	2710 ± 520	3500 ± 1125
N-Me-2-8	8670 ± 3340	>100,000	860 ± 110	900 ± 280
N-Me-2-9	6167 ± 2687	>100,000	872 ± 379	189 ± 28
N-Me-2-10	8175 ± 1970	>100,000	2493 ± 479	2115 ± 392
N-Me-2-11	6000 ± 4200	10,380 ± 2270	1050 ± 33	1160 ± 365
N-Me-2-12	8070 ± 2900	>100,000	4900 ± 3200	2420 ± 470
N-Me-2-13	4920 ± 1900	>100,000	1030 ± 109	2020 ± 350
N-Me-2-14	3440 ± 2890	>100,000	498 ± 67	740 ± 110
N-Me-2-15	2478 ± 1566	>100,000	1416 ± 922	605 ± 125
N-Me-2-16	5410 ± 2092	>100,000	1457 ± 731	1034 ± 129
N-Me-2-17	2340 ± 559	>100,000	1552 ± 845	1297 ± 100
N-Me-2-18	8440 ± 4495	>100,000	1850 ± 97	4590 ± 930

Table VIII

Functional Activity of N-Me-3 Library at the Mouse Melanocortin Receptors

Ref	mMC1R (nM)	mMC3R (nM)	mMC4R (nM)	mMC5R (nM)
NDP-MSH	0.0081 ± 0.001	0.063 ± 0.004	0.097 ± 0.016	0.078 ± 0.0084
Compound 1	64 ± 14	770 ± 175	4.0 ± 0.6	7.0 ± 0.40
N-Me-3-1	1350 ± 240	>100,000	613 ± 98	560 ± 75
N-Me-3-3	5090 ± 680	>100,000	1350 ± 270	810 ± 164
N-Me-3-4	6790 ± 1030	>100,000	1790 ± 880	782 ± 99
N-Me-3-10	19,700 ± 3670	>100,000	1498 ± 370	849 ± 320
N-Me-3-11	36,700 ± 22,900	>100,000	1320 ± 450	440 ± 69
N-Me-3-16	6030 ± 2080	>100,000	336 ± 59	290 ± 52