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Development of Macrocyclic Peptidomimetics Containing Constrained α,α -Dialkylated Amino Acids with Potent and Selective Activity at Human Melanocortin Receptors

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Abstract

We report the development of macrocyclic melanocortin derivatives of MT-II and SHU-9119, achieved by modifying the cycle dimension and incorporating constrained amino acids in ring-closing. This study culminated in the discovery of novel agonists/antagonists with an

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Accession Codes

The PDB code for the NMR structure of **8** in DPC solution is 6FCE. Authors will release the atomic coordinates and experimental data upon article publication.

Notes

The authors declare no competing financial interest.

DEDICATION

Dedicated to Prof. Victor J. Hruby on the occasion of his birthday.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmed-chem.8b00488.

Materials, analytical data and biological activity of **4–19**; ¹H NMR resonance assignments of **8** in DPC solution; NOE derived upper limit constraints of **8**; docking result of **1**/hMC3R complex (PDF)

Molecular formula strings and some data (CSV)

PDB coordinates of **1**/hMC3R (PDB)

PDB coordinated of **8**/hMC3R (PDB)

unprecedented activity profile by adding pieces to the puzzle of the melanocortin receptor selectivity. Finally, the resulting 19- and 20-membered rings represent a suitable frame for the design of further therapeutic ligands as selective modulators of the melanocortin system.

Graphical Abstract

INTRODUCTION

The proopiomelanocortin (POMC) gene, mostly expressed in the central nervous system (CNS) of humans, encodes its corresponding precursor polypeptide of 241 amino acids, which is subject to post-translational proteolytic cleavages.¹ From these processes the melanocortin peptides, α -, β -, and γ -melanocyte stimulating hormones (MSH) and the adrenocorticotropin hormone (ACTH), arise.¹ Such peptide hormones are the endogenous bioactive ligands for the human melanocortin receptors (*h*MCRs), and along with the melanocortin inverse agonists agouti signaling protein (ASiP) and agouti related protein (AgRP), all comprise the melanocortin system.^{1,2} To date, five G-protein-coupled receptor (GPCR) subtypes have been discovered and investigated for their ability to mediate several functions in the human body.^{3,4} Although the melanocortin system is well-known machinery involved in the regulation of several physiopathological functions,¹ structure–activity relationships (SARs) knowledge is still lacking in terms of structural requirements to reach fine modulation of *h*MCRs. In fact, extensive studies have been carried out,^{3,5,6} unveiling the core tetrapeptide His-Phe-Arg-Trp as a conserved and fundamental sequence for receptor activation.⁷ Furthermore, modifications of the α -MSH sequence have turned out to be successful for the development of other representative compounds, both linear and cyclic derivatives, such as the lead agonist MT-II (**1**, Chart 1) and antagonist SHU-9119 (**2**, Chart 1),^{8–12} albeit nonselective.

We have previously designed macrocyclic melanocortin analogues by a reduction of the ring cycle, by means of an alkylthioaryl bridge, from typically 23 members of **1** and **2** to 19 members (e.g., compound PG10N,¹³ **3** in Chart 1). In particular, these previously reported compounds have implied key features to attain improved selectivity, such as (i) reduced ring size and (ii) stabilized β -turn conformation. Therefore, herein we describe the design and synthesis of novel derivatives achieved by modifications of the cycle dimension and the incorporation of constrained amino acids in ring-closing. For this approach, we present the

synthesis of side-chain-to-tail cyclic **1** and **2** analogues containing 2-aminoisobutyric acid (Aib), 1-aminocyclobutane-1-carboxylic acid (Ac4c), 1-amino-cyclopentane-1-carboxylic acid (Ac5c), and 1-aminocyclohexane-1-carboxylic acid (Ac6c) residues, all involved in a side-chain-to-tail cyclization with Asp and Glu acidic residues (**4–19**, Chart 2). This strategy has been pursued to investigate the influence of the cycle structure and to understand the effects of incorporation of constrained Aib-derived amino acids on the conformation and interaction with melanocortin receptors. This study has led to the discovery of compounds with unprecedented profiles of activity and selectivity and has improved SAR knowledge regarding the melanocortin system.

RESULTS AND DISCUSSION

Design

Extensive SAR studies performed on melanocortin ligands pointed out that conformational restriction can contribute to receptor selectivity.^{14–16} Starting from the exciting results obtained with **3** (Chart 1) and with the aim to discover new potent and selective ligands, we have designed novel macrocyclic compounds in which an α,α -disubstituted amino acid has replaced the alkylthioaryl bridge at the N-terminus (Chart 2). With α -hydrogen exchanged with a methyl group, the Aib residue is almost invariably restricted to ϕ , ψ values of either $-60^\circ (\pm 20^\circ)$ and $-30^\circ (\pm 20^\circ)$ or $60^\circ (\pm 20^\circ)$ and $30^\circ (\pm 20^\circ)$.¹⁷ Ac4c–Ac6c not only adopt similar ϕ , ψ angle constraints of Aib but have additional constraints to the N–C α –C' (τ) bond angle. For example, Ac4c was shown to have a wider than normal τ angle of 114.7° .¹⁸ It is expected to have a decreasing trend for the τ bond angle for Ac4c–Ac6c. These structural constraints have been introduced to further restrict the conformation of the macrocyclic compounds, which in turn may improve potency and selectivity. Then, ring closure is obtained by a lactam bridge formed by the carboxyl group of side chain of a Glu or Asp residue at position 10 and the amino group of residue 5. Following this strategy, we have designed and synthesized two series of macrocyclic compounds containing Asp (series A, **4–11**) or Glu (series B, **12–19**). The resulting macrocyclic peptidomimetics, possessing a 19- or 20-membered ring, have conserved the melanocortin core sequence His-DPhe/DNal(2')-Arg-Trp (Chart 2).

Chemistry

Peptides **4–19** have been manually synthesized by solid-phase peptide synthesis (SPPS) using conventional 9-fluorenylmethoxycarbonyl (Fmoc) chemistry.¹⁹ The synthetic steps of selective allyl ester hydrolysis and intramolecular coupling have been performed by microwave (μ W)-assisted SPPS, following slightly modified protocols elsewhere reported.^{20,21} Upon cyclization and cleavage from the solid support, crude peptides have been purified by reverse-phase high pressure liquid chromatography (RP-HPLC).

Biological Evaluation

All synthesized compounds (**4–19**) have been evaluated for their binding affinities to the *h*MCRs 1, 3, 4, and 5 in competitive binding assays using the radiolabeled ligand [¹²⁵I]-NDP- α -MSH and for their agonist potency in cAMP assays employing the HEK293 cells

expressing those receptors. Data of the most representative analogues are collected in Table 1 (for the whole library of compounds see Table S2 in Supporting Information).

Overall, binding data indicate that affinities of the new compounds toward *h*MCRs are dependent on macrocycle ring size. In particular, the compounds of series A (**4–11**) show medium/high nM range or null affinity for all *h*MCRs ($IC_{50} > 70$ nM) except for the *h*MC3R. In fact, **4**, **5**, **7–11** strongly bind to the *h*MC3R ($IC_{50} < 20$ nM). This leads to some selective ligands (**7–11**) at the *h*MC3R. Exceptions to the above observation are compound **6**, which does not bind to any *h*MCR, and compounds **4** and **5**, which have high and moderate affinity also for *h*MC4R. Increasing the ring size to 20 atoms (series B) leads to **12–19**, which show low affinities for *h*MC1R ($IC_{50} > 40$ nM). In regard to *h*MC3R, DPhe⁷ containing compounds generally show lower affinity compared to their corresponding 19-membered cycles (**12** vs **4**, **16** vs **8**, **18** vs **10**). Conversely, DNal⁷ derivatives (**13**, **15**, **17**, **19**) retain similar or improved affinity for *h*MC3R compared to their lower homologs. For *h*MC4R, compounds **14**, **16**, and **18** show no binding at this receptor. Finally, considering *h*MC5R, some DNal⁷ derivatives (**13**, **15**, **19**) show good affinities ($IC_{50} = 12–21$ nM), significantly improved compared to the series A homologs.

Considering the functional activity measured in terms of cAMP levels, all the compounds are full agonists at *h*MC1R as their parents **1** and **2**, but their potency is always lower than **1** ($EC_{50} > 8$ nM). For the *h*MC3R, all the analogs containing a DPhe⁷ are full agonists as the parent **1**. Potencies on this receptor depend on the ring size, with series B derivatives (**12**, **14**, **16**, **18**) being generally more potent than the corresponding compounds belonging to series A. Among those, **14** and **16** have low nM EC_{50} values (about 1 nM) similar to **1**. In particular, **14** is found to be an interesting agonist at *h*MC3R but without selectivity with respect to the *h*MC1R (1:1) and with only modest selectivity when compared to *h*MC5R (1:7). Interestingly, **8** and **16** are selective agonists for the *h*MC3R in terms of binding and activity when compared to *h*MC4R and *h*MC5R. In particular, **16** shows a nM binding at *h*MC1R and *h*MC3R and no binding at the 4 and 5 *h*MCRs. This compound represents a highly potent full agonist at the *h*MC1R and *h*MC3R with EC_{50} values of 8.6 and 1.0 nM, respectively. The analogs containing DNal⁷ are often partial agonists at *h*MC3R with the notable exceptions of **15** and **19**, which behave as full antagonists. In this context, **13** and **17**, which differ from **15** and **19** only due to the α,α -amino acid at position 5, are potent partial agonists at *h*MC3R (EC_{50} of 19 and 5 nM, respectively). Thus, the constrained amino acid at position 5 influences the activity profile of the **2**-derived (i.e., DNal containing) analogs and similar results are also observed for the *h*MC4R. In fact, **15** and **19** are full antagonists at *h*MC4R while **17** is a partial agonist and **5** and **13** are full agonists at *h*MC4R.

Considering the activity on *h*MC5R, many compounds that show some affinity for this receptor have full agonist activity resembling the parent peptides **1** and **2**. In this context, **12** is a potent agonist ($EC_{50} < 15$ nM) at all the *h*MCRs. Notable exceptions are **13**, **15**, and **19**, which are ligands with high affinity and full antagonism at *h*MC5R. In particular, **13** is a potent antagonist at *h*MCR5, a full agonist at *h*MC1R and *h*MC4R, and a partial agonist at *h*MC3R, which is a unique activity profile for a melanocortin agent. Moreover, **15** and **19** containing DNal(2') residue in position 7 showed an unexpected but exceptional profile of

activity resulting in potent antagonists at all *h*MCRs except for the *h*MC1R. These compounds represent the first macrocyclic peptides endowed with full antagonist activity at the 3, 4, and 5 *h*MCRs.

According to the bioassay results, α,α -diakylated amino acids played an important role for modulating selectivity. In series A, the Ac6c containing peptides **10** and **11** are 4–7 times more potent at *h*MC1R than the Aib containing compounds **4** and **5**. The Ac4c containing peptides **6** and **7**, on the other hand, are 40–107 times less potent at *h*MC4R than the Aib containing compounds **4** and **5**. In series B, the Ac6c containing peptides **18** and **19** become 4–49 times less potent at *h*MC1R than the Aib containing compounds **12** and **13**. More importantly, Ac4c and Ac6c have enhanced the antagonist activity of the DNal(2') containing peptides **15** and **19** at *h*MC3R and *h*MC4R. These modulations on potency are probably due to different presentations of the His-DPhe/DNal(2')-Arg-Trp pharmacophore as a result of the ϕ , ψ , and τ angle constrains.

NMR Analysis

Detailed conformational analysis by solution NMR was performed on **8**. Compound **8** was chosen because it is a high affinity ($IC_{50} = 10$ nM) ligand at *h*MC3R with a selectivity of at least 25 times over all the other *h*MCRs. It is also a potent *h*MC3R full agonist ($EC_{50} = 30$ nM) with high functional selectivity vs the other central *h*MCRs (4 and 5). Moreover, belonging to the series A, **8** was designed to be also highly conformationally constrained. A whole set of one-dimensional (1D) and two-dimensional (2D) NMR spectra in a 200 mM aqueous solution of dodecylphosphocholine (DPC) were collected for **8**. DPC micelle solutions were used since they are membrane mimetic environments and are largely used for conformational studies of peptide neurotransmitters and hormones.^{22,23} All NMR parameters are reported in Table S3, Supporting Information. NMR-derived constraints obtained for **8** were used as the input data for a simulated annealing structure calculation. NOESY spectra of **8** showed diagnostic NOEs consistent with turn/helical structures (Table S4, Supporting Information). The restrained simulated annealing calculation gave an ensemble of structures showing a type II' β -turn about residues DNal(2')⁷-Arg⁸, followed by a short 3_{10} -helix along residues Trp⁹ and Glu¹⁰ (Figure 1). The complete ensemble of structures fulfilled the NOE restraints, with no violations exceeding 0.5 Å. Considering the side chains, DPhe⁷, Arg⁸, and Trp⁹ show a clear preference for *trans*, *g*⁺, and *g*⁻ and orientations, respectively. His⁶ is less defined, but there is a predominance of the *g*⁻ orientation (about 60% of the calculated structures). Spatial proximity of the Arg⁸ side chain to the indole of Trp⁹ is in accordance with the significant upfield shift of the proton signals of the Arg⁸ residue.

Docking

8 was also docked against an *h*MC3R model,²² using the program AUTODOCK.²⁴ This gave a high populated low energy cluster for the **8**/*h*MC3R complex, whose best scored pose is shown in Figure 2. The predicted binding site is placed among TM3–7, EL1–3 (Figure 2a). Main interactions between the peptide and *h*MC3R are shown in Figure 2b. For comparison purpose, NMR structure of **1**²⁵ was also docked to the *h*MC3R model. Docking results are reported in Figure S1 of the Supporting Information. **1** location in the docking

best pose is very similar to **8**. The main difference is the position of Trp⁹ side chain in the two complexes, which could be responsible of the different activity profiles of the two compounds. This issue requires further investigation.

CONCLUSIONS

We have developed novel melanocortin ligands by reducing the cycle dimension of known *h*MCRs agonist and antagonist and introducing α,α -disubstituted constrained amino acids. Among the novel compounds, we have obtained (i) highly potent agonists at the *h*MC3R (**8**, **10**, **12**, **14**, **16**, **18**), some of which were also highly selective toward this receptor (**8** and **10**); (ii) a potent agonist at all the *h*MCRs (**12**); (iii) a potent antagonist at *h*MC5R, full agonist at *h*MC1R and *h*MC4R, and partial agonist at *h*MC3R (**13**), which is a unique activity profile for a melanocortin agent; (iv) potent antagonists at the 3, 4, and 5 *h*MCRs and agonists at *h*MC1R (**15** and **19**) which is again an unprecedented activity profile for *h*MCRs. All these compounds enlarge our structure–activity relationships knowledge for the melanocortin ligands and offer new tools for pharmacological studies. Moreover, the activities shown by these compounds demonstrate that the new cyclic scaffolds with 19- and 20-membered ring are suitable for the development of *h*MCRs ligands and can be employed for further development of melanocortins. Finally, NMR-derived structure of **8** and its docking model against *h*MC3R were calculated. Overall, the results will help to develop novel peptide and non-peptide analogues acting on this target which is of overwhelming importance in different pathological conditions in which melanocortin system play an important role.

EXPERIMENTAL SECTION

General Procedures

Microwave irradiation was performed on a Biotage Initiator⁺ apparatus on the high-absorption level; temperature was monitored automatically. LC–MS analyses were performed on a LC–MS instrument from Agilent Technologies equipped with an analytical C18 column and 6110 quadrupole, in positive electrospray ionization (ESI) mode, to confirm that difficult couplings had achieved >90% conversion. HRMS measurements were recorded on a LTQ Orbitrap mass spectrometer in positive ESI mode, and proton adducts [*M* + H]⁺ were used for empirical formula confirmation.

Purification of peptides **4–19** was performed by RP-HPLC (Shimadzu preparative liquid chromatograph LC-8A) equipped with a preparative column (Phenomenex Kinetex C18 column, 150 mm × 21.2 mm, 5 μ m, 100 Å) using linear gradients of MeCN (0.1% TFA) in water (0.1% TFA), from 10% to 90% over 20 min, with a flow rate of 10 mL/min and UV detection at 220 nm. Final products were obtained by lyophilization of the appropriate fractions after removal of the MeCN by rotary evaporation. Peptides **4–19** were analyzed by analytical RP-UHPLC (Shimadzu Nexera liquid chromatograph LC-30AD) equipped with a C18-bonded reverse-phased column (Phenomenex Kinetex, 150 mm × 4.6 mm, 2.6 μ m, 100 Å) using gradient elution of two different solvent systems [gradient 1 of MeCN (0.1% TFA) in water (0.1% TFA) from 10% to 90% over 15 min; gradient 2 of MeOH (0.1% TFA) in water (0.1% TFA) from 10% to 90% over 15 min; flow rate = 1 mL/min; diode array UV–vis detector; see Supporting Information]. Such analytical methods assessed for purity of

peptides **4–19** > 99% (see Table S1, Supporting Information), and their correct molecular weights were also confirmed by HRMS spectrometer.

Peptide Synthesis

The synthesis of macrocyclic peptides **4–19** was performed in a stepwise fashion via solid-phase peptide synthesis (SPPS), as elsewhere reported.¹⁹ In particular, each peptide sequence was assembled on a Rink amide resin (0.2 mmol from 0.74 mmol/g of loading substitution) as solid support placed into a plastic syringe tube equipped with Teflon filter, stopper, and stopcock. The resin, stored as Fmoc-protected, was preswollen in DMF for 20 min, then treated with 20% piperidine in DMF solution (5 min $\times$ 1, 25 min $\times$ 1) to remove the Fmoc group. The first peptide bond formation by the coupling with the first residue [Fmoc-Asp(OAll) or Fmoc-Glu(OAll)] (3 equiv) was achieved by adding 3-fold excess of 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt) in the presence of a 6-fold excess of *N,N*-diisopropylethylamine (DIEA). The Fmoc deprotection was carried out as described above. After each coupling and Fmoc-deprotection step, the peptide resins were washed with DMF (2 mL $\times$ 3), DCM (2 mL $\times$ 3), and DMF (2 mL $\times$ 3), and reactions were monitored by the colorimetric Kaiser test for the detection of solid-phase bound primary amines.²⁶

The peptide resins carrying the allyl esters of aspartic and glutamic residues in C-terminal were hydrolyzed by a slightly modified procedure elsewhere described.^{20,21} In particular, the resins were washed with DCM (2 mL $\times$ 3), suspended in a solution of Pd(PPh₃)₄ (0.15 equiv) and *N,N'*-dimethylbarbituric acid (NDMBA) (7 equiv) in 3:2 dry DCM/DMF (v/v), and placed in a 20 mL μ W reaction vessel. The vessel was sealed and heated to 40 °C using μ W irradiation for 5 min. The vessel was opened, and the reaction mixture was transferred to a plastic syringe tube. The resins were filtered, washed with DMF (2 mL $\times$ 3) and DCM (2 mL $\times$ 3), and the allyl-deprotection procedure was repeated under the same conditions. The resins were filtered, washed with DMF (2 mL $\times$ 3), a 0.5% solution of sodium *N,N*-diethyldithiocarbamate in DMF (30 min $\times$ 2), and DCM (2 mL $\times$ 3). After complete removal of the allyl group from the peptide was ascertained by LC–MS of the residue from cleavage of an aliquot of resin (5 mg), the N-terminal Fmoc group was finally removed, as above-described. The released amines were thus coupled to acids by using PyAOP (2 equiv) and HOAt (2 equiv), which are known effective coupling reagents for the intramolecular amide bond formation,^{27,28} in the presence of DIEA (4 equiv). The reaction was carried out in a sealed 20 mL μ W reaction vessel, heated to 45 °C under μ W irradiation for 5 min. The vessel was opened, and the reaction mixture was transferred to the plastic syringe tube. The peptide-resin was thoroughly washed with DCM (5 $\times$ 2 mL) and dried under argon.

Peptides **4–19** were released from the resin and the protecting groups cleaved simultaneously by means of a reaction cocktail consisting of 95:2.5:2.5 TFA/TIS/H₂O (v/v/v) at rt for 3 h. The resin was removed by filtration and crude peptides were recovered by precipitation with chilled anhydrous Et₂O as white to pale beige- colored amorphous solids.

Biological Activity Assays

Competitive binding assays with [125 I]-NDP-MSH and cAMP assays were performed on HEK293 cells stably expressed human melanocortin receptors (*h*MC1R, *h*MC3R, *h*MC4R, *h*MC5R). The methods used previously described protocols.^{29–34}

NMR Spectroscopy

The samples for NMR spectroscopy were prepared by dissolving the appropriate amount of **8** in 0.54 mL of $^1\text{H}_2\text{O}$ (pH 5.5), 0.06 mL of $^2\text{H}_2\text{O}$ to obtain 2 mM peptide and 200 mM DPC- d_{38} . NMR spectra were recorded on a Varian INOVA 700 MHz spectrometer equipped with a z-gradient 5 mm triple-resonance probe head. 1D and 2D NMR spectra were recorded and processed as described in the Supporting Information.

Structure Calculation

The NOE-based distance restraints were obtained from NOESY spectrum of **8** collected with a mixing time of 100 ms. The NOE cross peaks were integrated with the XEASY³⁵ program and were converted into upper distance bounds using the CALIBA program incorporated into the program package DYANA.³⁶ Only NOE derived constraints were considered in the annealing procedures (Table S4, Supporting Information). Restrained simulated annealing was performed using the Discover module of the InsightII program. More details are reported in the Supporting Information.

Receptor Models and Docking

Three-dimensional structure model of *h*MC3R was generated by I-TASSER³⁷ server for protein structure and function prediction, as previously reported.²² The initial pose for the *h*MC3R/**8** or *h*MC3R/**1** complexes was generated by docking the lowest energy conformers of **8** and **1** obtained by NMR to the *h*MC3R model using the program AUTODOCK 4.0.²⁴ The side chains of His⁶, DPhe⁷, Arg⁸, and Trp⁹ of **8** and **1** were considered flexible in the docking procedure. Refinement of lowest energy pose of *h*MC3R/**8** and *h*MC3R/**1** complexes was achieved by in vacuo energy minimization with the Discover algorithm using the steepest descent and conjugate gradient methods until a rmsd of 0.05 kcal/mol per Å was reached. The backbone atoms of the TM and IL domains of the *h*MC3R were held in their position; the ligands and ELs were free to relax.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS USED

1D one-dimensional

2D	two-dimensional
Ac4c	1-aminocyclobutanecarboxylic acid
Ac5c	1-aminocyclopentane-carboxylic acid
Ac6c	1-aminocyclohexanecarboxylic acid
Aib	2-aminoisobutyrric acid
DIEA	<i>N,N</i> -diisopropylethylamine
DPC	dodecylphosphocholine
EL	extracellular loop
HBTU	2-(1 <i>H</i> -benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexa-fluorophosphate
hMCR	human melanocortin receptor
HOBt	1-hydroxybenzotriazole
MSH	melanocyte stimulating hormone
Nal(2')	2-naphtylalanine
NDMBA	<i>N,N'</i> -dimethyl-barbituric acid
POMC	proopiomelanocortin
RP-HPLC	reversed-phase high performance liquid chromatography
TM	transmembrane domain

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Figure 1.
Lowest energy conformers of compound **8** (PDB code 6FCE).

Figure 2.

(a) *hMC3R* model²² complexed with **8**. Heavy atoms of **8** (carbon, green; nitrogen, blue; oxygen, red; sulfur, yellow). Receptor backbone is represented in gray ribbon. (b) **8** within the binding pocket of *hMC3R*. Hydrogen bonds are represented with dashed lines.

Chart 1.
Chemical Structures and Ring Cycle Dimension of MT-II (1), SHU-9119 (2), and PG10N (3)

Biological Activities of the More Representative Macrocyclic Peptides at the *h*MC1R and 3–5 *h*MCRs

Table 1

	<i>h</i> MC1R				<i>h</i> MC3R				<i>h</i> MC4R				<i>h</i> MC5R			
	IC ₅₀ ^a (nM)	EC ₅₀ ^b (nM)	Act % ^c	IC ₅₀ ^a (nM)	EC ₅₀ ^b (nM)	Act % ^c	IC ₅₀ ^a (nM)	EC ₅₀ ^b (nM)	Act % ^c	IC ₅₀ ^a (nM)	EC ₅₀ ^b (nM)	Act % ^c	IC ₅₀ ^a (nM)	EC ₅₀ ^b (nM)	Act % ^c	Act % ^c
1	1.2 ± 0.2	1.8 ± 0.2	100	1.2 ± 0.2	1.5 ± 0.2	100	1.1 ± 0.2	2.8 ± 0.4	100	7.5 ± 0.5	3.3 ± 0.6	100	7.5 ± 0.5	3.3 ± 0.6	100	100
8	440 ± 6	28 ± 4	100	10 ± 1	30 ± 4	100	260 ± 23	280 ± 7	100	NB ^e	NA ^d	0	NB ^e	NA ^d	0	0
10	240 ± 10	13 ± 2	100	11 ± 1	33 ± 3	100	160 ± 9	10 ± 1	96	>1000	NA ^d	0	>1000	NA ^d	0	0
12	320 ± 32	15 ± 4	100	57 ± 6	12 ± 4	100	74 ± 3	5.3 ± 0.4	100	440 ± 115	14 ± 1	92	440 ± 115	14 ± 1	92	92
13	470 ± 10	13 ± 2	100	27 ± 5	19 ± 2	60	370 ± 41	18 ± 1	100	12 ± 1	NA ^d	0	12 ± 1	NA ^d	0	0
15	120 ± 6	41 ± 4	100	2.2 ± 0.2	NA ^d	0	71 ± 2	NA ^d	0	21 ± 1	NA ^d	0	21 ± 1	NA ^d	0	0
19	100 ± 12	631 ± 25	100	4.4 ± 0.1	NA ^d	0	59 ± 4	NA ^d	0	20 ± 3	NA ^d	0	20 ± 3	NA ^d	0	0

^aIC₅₀ = concentration of peptide at 50% specific binding (*N* = 4).^bEC₅₀ = effective concentration of peptide that was able to generate 50% maximal intracellular cAMP accumulation (*N* = 4).^cAct % max is ratio of the highest cAMP level triggered by peptides over the highest cAMP level triggered by MT-II (1). The peptides were tested at a range of concentrations from 10^{−10} to 10^{−6} M.^dNA: 0% cAMP accumulation at 10^{−5} M.^eNB: no binding at 10^{−5} M.