4-{(*2R*)-[3-Aminopropionylamido]-3-(2,4-dichlorophenyl)propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]phenyl}piperazine as a Potent and Selective Melanocortin-4 Receptor Antagonist−Design, Synthesis, and Characterization

Chen Chen,* Joseph Pontillo, Beth A. Fleck, Yinghong Gao, Jenny Wen, Joe A. Tran, Fabio C. Tucci, Dragan Marinkovic, Alan C. Foster, and John Saunders

Neurocrine Biosciences, Inc. 12790 El Camino Real, San Diego, California 92130

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Recent studies have demonstrated that melanocortin-4 receptor (MC4R) antagonists can prevent weight loss in tumor-bearing mice, which indicates clinical usage for the treatment of cachexia. In our efforts to develop potent and selective antagonists of the human MC4R, we designed piperazinebenzylamines bearing a 2,4-dichlorophenylalanine, by utilizing information derived from structure−activity relationships of MC4R agonists and mutagenesis results of the MC4R and peptide ligands. On the basis of known MC4R agonists such **6**, we successfully synthesized potent MC4R antagonists exemplified by **10**, which possesses a K_i value of 1.8 nM in binding affinity. **10** does not stimulate cAMP release in HEK 293 cells expressing the human MC4 receptor at 10 μM concentration. It was demonstrated by Schild analysis that **10** was a competitive functional antagonist with a pA_2 value of 7.9 in the inhibition of α-MSH-stimulated cAMP accumulation. **10** also penetrated into the brain when dosed intravenously in rats.

Introduction

G-protein-coupled receptors (GPCRs) are a family of structurally related membrane-bound proteins that play a central role in the recognition and signal transduction of hormones and neurotransmitters. GPCRs share general structural motifs, including seven transmembrane helices connected by intra- and extracellular loops, an extracellular amino terminus, and a cytoplasmic carboxyl terminus.[1] GPCRs are activated by light, small molecules such as histamine, peptides, and proteins.[2] Although many of them are considered to be drug targets, GPCRs activated by peptides and proteins,[3] due to a lack of structural information, have been particularly challenging to medicinal chemists to design nonpeptide small-molecule agonists or antagonists of these receptors.[3b] The recent resolution of the crystal structure of bovine rhodopsin shines some light on GPCR research, since it provides the first three-dimensional structure of any GPCR,[4] thereby making it possible to analyze experimental data of other GPCRs from a more informed and structural perspective.[1b,5] Because there are many conserved residues in the transmembrane domains across the class A GPCRs,[6] the crystal structure of rhodopsin should offer a good template for computational modeling of many of these receptors.[7] In addition, site-directed mutagenesis has made it possible to characterize the ligand interaction sites of GPCRs.[8] A binding pocket in the transmembrane domain of several class A GPCRs, including the adrenergic and neurokinin receptors,[9] has been identified via mutation studies.

Five subtypes of melanocortin receptors, MC1−5R, that belong to the class A GPCR superfamily have been identified and cloned.[10] The MC1R regulates skin pigmentation and the immune system. The MC2R (ACTH receptor) controls steroid production. The MC3R might be involved in regulation of central sexual behavior, the MC4R controls feeding behavior, and the MC5R has a role for regulating exocrine gland secretion.[11] The melanotropin peptides are the natural ligands for the melanocortin receptors and consist of the melanocyte-stimulating hormones α-, β-, and γ-MSH and the adrenocorticotropin ACTH. In addition, two endogenous antagonists, agouti-protein and agouti-related protein (AgRP), have been identified.[12] All the melanotropin peptides possess a His-Phe-Arg-Trp (HFRW) motif, which has been proven to be crucial for activation of the melanocortin receptors.[13] Agouti-protein and AgRP, on the other hand, have an Arg-Phe-Phe motif located in a loop known to interact with the melanocortin receptors[14] and believed to be important for functional antagonism.[15]

While current research of the melanocortin system has focused on MC4R agonists for the possible treatment of obesity,[16] recent studies have showed that peptide MC3/MC4 receptor antagonists effectively prevent tumor-bearing mice from reduction of food intake and, more importantly, weight loss.[17] Studies have also shown that MC4R knock-out mice resist tumor-induced loss of lean body mass.[17a] These positive results in animal models indicate a potential clinical treatment of cachexia by using a melanocortin antagonist, which is significant since this wasting syndrome occurs in a large population of cancer patients.[18] Very recently a small molecule MC4R antagonist ML253764 (**2**) has been shown, upon peripheral administration in tumor-bearing mice, to protect against weight loss.[19] However, while this molecule displays moderate potency at the human MC4R (IC_{50} = 106 nM in inhibition of cAMP production), its activity at the MC3R is not clear.

* Corresponding author. Telephone: 858-617-7600. Fax: 858-617-7967. E-mail: cchen@neurocrine.com.

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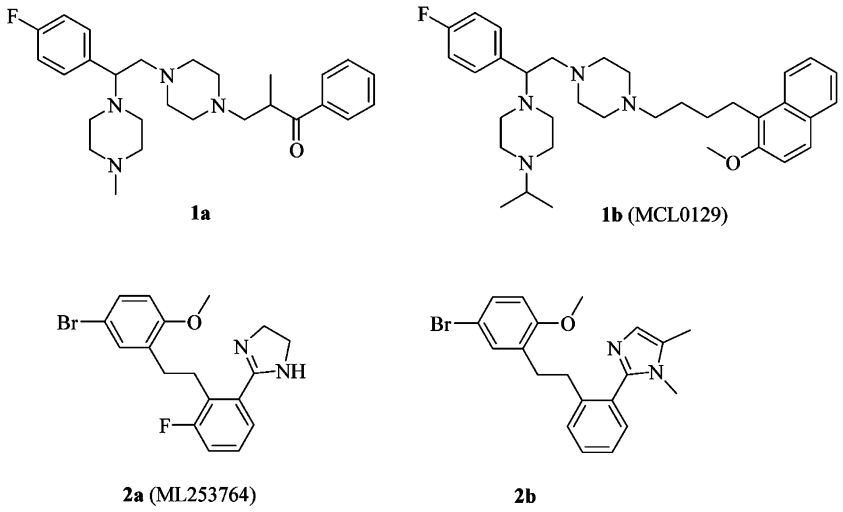


Figure 1. Small molecule antagonists of the melanocortin-4 receptor.

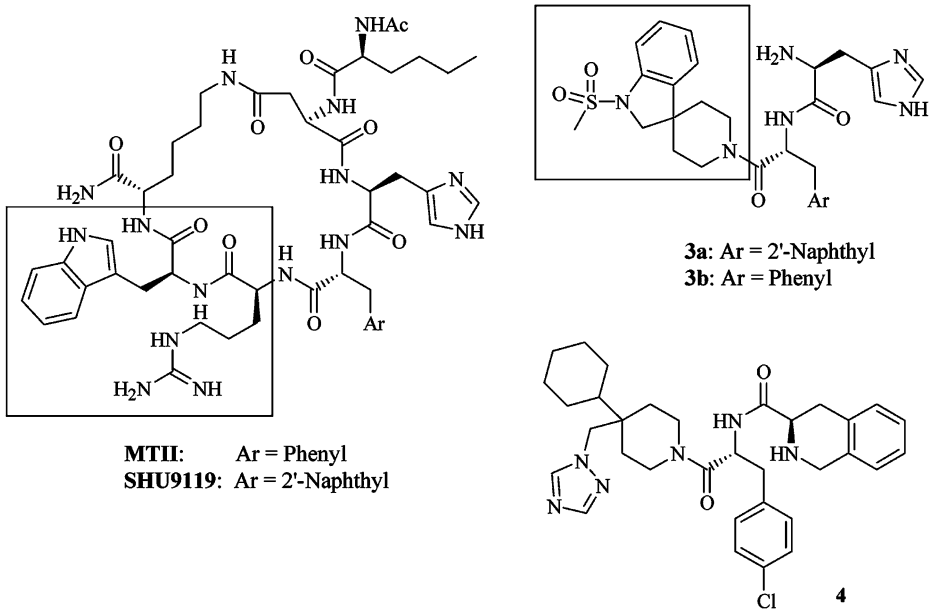


Figure 2. Chemical structure of MTII, SHU9119, and small molecule MC4R ligands **3** and **4**.

Small molecule antagonists of the melanocortin-4 receptor have been discovered recently (Figure 1). Compound **1a**, first reported to be an inhibitor of AgRP binding to the MC4R (IC_{50} = 52 nM), also exhibits moderate inhibition (IC_{50} = 220 nM) of NDP-MSH binding and reduces α-MSH-stimulated cAMP production at high concentration.[20] MCL0129 (**1b**), which has structural similarity to **1a**, binds to the MC4R with high affinity (K_i = 7.9 nM). Interestingly, **1b** has shown anxiolytic-like and antidepressant-like activities in several animal models.[21] The benzamidine **2a** (ML253764) is reported to have a K_i value of 160 nM in binding affinity at the MC4R.[19] A series of imidazoles such as **2b** (K_i = 180 nM) as antagonists of the MC4R has also been reported.[22]

On the other hand, several agonists from different chemical classes have been discovered since compound **4** (EC_{50} = 1.2 nM)[23] was disclosed as the first potent and selective small molecule MC4R agonist in 2002.[24] **4** is reported to be developed from the initial leads **3a,b**,[23] which are in turn designed by mimicking the His-D-Phe-Arg-Trp motif of MT-II by replacing the Arg-Trp residues with a privileged structure for GPCRs (Figure 2).[25] Interestingly, the triazole moiety of **4** can be replaced by a *N*-methyltetrazole (**5b** or **5c**, Figure 3) but not tetrazole itself (**5a**). Thus, **5b** has an EC_{50} of 0.6 nM, while **5a** is a poor MC4R agonist (EC_{50} = 771 nM).

Recently, a series of substituted phenylpiperazines such as **6** (**6a**: EC_{50} = 110 nM), which are characterized as MC4R-selective agonists, has been reported from our laboratory (Figure 3).[24d] We then optimized this series into highly potent derivatives such as **8b** (EC_{50} = 4.7 nM).[26] Because of the potential clinical importance for the possible treatment of cancer cachexia, and anxiety/depression, we are interested in the development of potent and selective small molecule MC4R antagonists. Herein we report our design, synthesis, and characterization of 4-{(2*R*)-[3-aminopropionylamido]-3-(2,4-dichlorophenyl)propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]-phenyl}piperazine **10** as such a molecule (see Scheme 1). Our design strategies are based upon information derived from structure−activity relationships of known small molecule and peptide MC4R agonists, in combina-

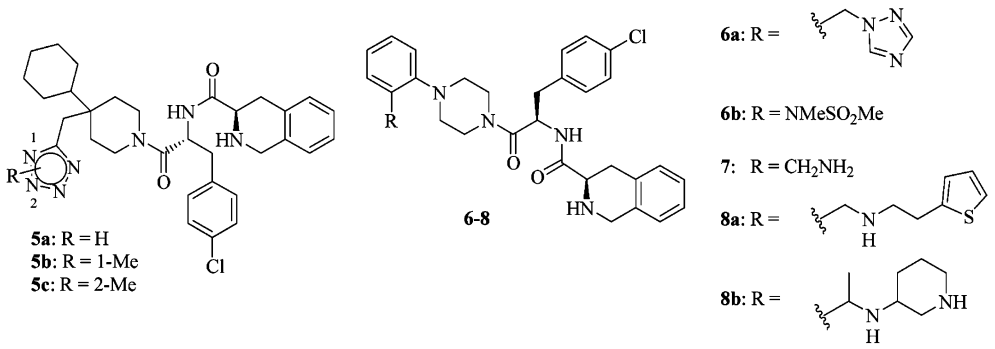
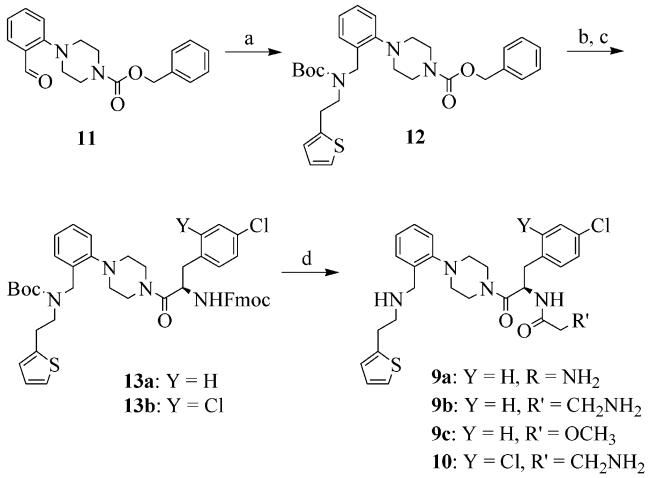


Figure 3. Chemical structure of some MC4R ligands with a Tic group.

Scheme 1[a]



[a] Reagents and conditions: (a) (i) 2-(2-thienyl)ethylamine/NaBH(OAC)$_3$/DCM; (ii) (Boc)$_2$O, 82%; (b) Pd/C−H$_2$/NH$_3$/MeOH, 35%. (c) *N*-Fmoc-(*R*)-(4-Cl)Phe-OH or *N*-Fmoc-(*R*)-(2,4-Cl)Phe-OH/EDC/HOBt/DCM; (d) (i) Et$_2$NH/DCM, 87%; (ii) R′CH$_2$COOH/EDC/HOBt/DCM; (iii) HCl/dioxane, 34−65%.

tion with site-directed mutagenesis of the MC4R and melanotropin peptides.

Chemistry

The syntheses of **9** and **10** started from the protected piperazinebenzaldehyde **11**, which was subjected to a reductive amination with 2-(2-thienyl)ethylamine and sodium triacetoxyborohydride, followed by Boc protection of the resulting secondary amine to give the intermediate **12** in 82% yield. Removal of the Cbz protecting group of **12** by palladium-catalyzed hydrogenolysis provided a free amine, which was coupled under standard peptide coupling conditions (EDC/HOBt) with *N*-FMOC-D-(4-Cl)Phe-OH or *N*-FMOC-D-(2,4-Cl)Phe-OH to afford the amides **13a** or **13b**, respectively. After FMOC-deprotection of **13** with diethylamine, the resulting free amines were coupled with *N*-Boc-glycine, *N*-Boc-β-alanine, or methoxyacetic acid to give the final compounds **9a**−**c** and **10** after TFA-deprotection.

Results

In Vitro Binding and Agonist and Antagonist Activities at the Human MC4 Receptor. The synthesized compounds were tested for their ability to compete with radiolabeled NDP-MSH in HEK 293 cells stably expressing the human MC4R. These compounds were also measured for their ability to stimulate cAMP production in the same cell lines as previously described,[27] and these results are depicted in Table 1.

Table 1. Summary of Binding and Function of 4 and 6−10 at the Human MC4 Receptor[a]

compd	K_i (nM)[b]	EC_{50} (cAMP, nM)[c]	IA (%)[c,d]
3a	(108)	n/a	n/a
3b	n/a	(530)	(118)
4	9.8 ± 2.1 (1.2)	1.45 ± 0.6 (2.1)	100 (97)
5a	(238)	(771)	(79)
5b	(0.3)	(0.6)	(103)
5c	(1.2)	(9.6)	(87)
6a	270 ± 65	110 ± 61	105
7	380 ± 10	890 ± 160	131
8a	10.8 ± 2.3	290 ± 48	105
8b	6.4 ± 1.4	4.77 ± 0.7[f]	100
9a	44.0 ± 1.1	580 ± 81	102
9b	20.5 ± 0.6		14
9c	69.5 ± 0.8		16
10[e]	1.8 ± 0.2		3

[a] The human melanocortin-4 receptor stably expressed in HEK 293 cells. [b] Values in parentheses are IC_{50} (nM) cited from ref 23. [c] Values in parentheses are cited from ref 23. [d] Intrinsic activity (IA): percentage of the maximal cAMP levels by α-MSH stimulation. [e] pA_2 = 7.9 in inhibition of α-MSH-stimulated cAMP production. [f] An EC_{50} of 3.8 nM was obtained on CHO cells.

Compound **4** had a K_i value of 9.8 nM in our competition binding assay (an IC_{50} of 1.2 nM was reported)[23] and an EC_{50} value of 1.45 nM, which matches well with the reported value of 2.1 nM. The discrepancy in K_i value could be caused by different assay conditions. The parent primary benzylamine **7** had moderate affinity and agonist potency. Incorporation of a 2-(2-thienyl)-ethyl side chain on **7** resulted in **8a** with much better binding affinity (35-fold increase) but only 3-fold increase in agonist potency. Replacement of the *R*-1,2,3,4-tetrahydroisoquinolin-3-ylcarbonyl (Tic) moiety of **8a** with a glycine gave compound **9a** with 4-fold reduction in affinity (K_i = 44 nM) but little change in agonist potency and efficacy. Its close analogue **9b** from β-alanine possessed slightly increased affinity (K_i = 20.5 nM), and the methoxyacetamide **9c** (K_i = 69.5 nM) displayed about 2-fold reduction. However, unlike **8a** and **9a**, compounds **9b** and **9c** exhibited very low efficacy in stimulation of cAMP release at 10 μM concentration (14−16% of α-MSH maximal level). Using a 2,4-dichlorophenyl group to replace the 4-chlorophenyl moiety of **9b** not only increased binding affinity by over 10-fold but also completely diminished agonist efficacy (Table 1). Thus, **10** showed no significant stimulation of cAMP release at 10 μM concentration (3% of α-MSH maximal level). **10** was also demonstrated by a Schild analysis to function as a competitive antagonist with a pA_2 value

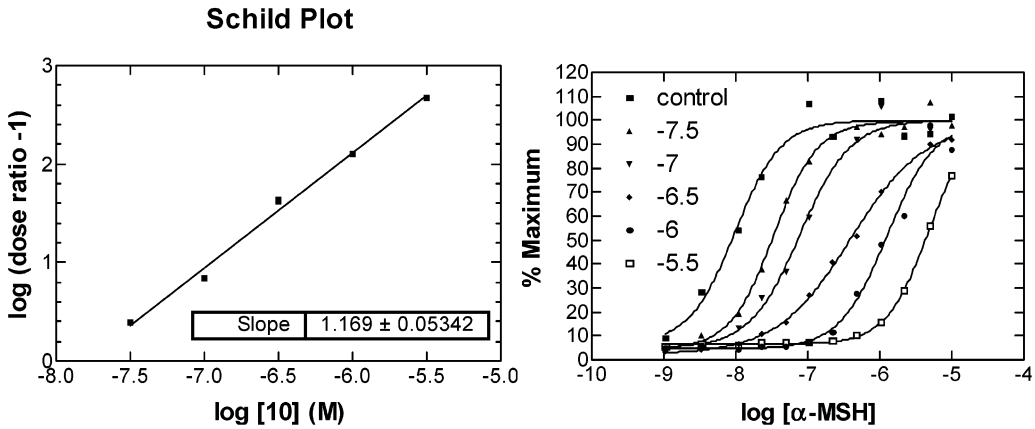


Figure 4. Dose−response curves and Schild analysis of compound **10** in cAMP assay.

Table 2. Binding Affinity (K_i, NM) of 8−10 at the Human Melanocortin Receptor Subtypes[a]

compd	hMC1	hMC3	hMC4	hMC5
7	(27%)[b]	(34%)b	380	2300
9a	(50%)[b]	1900	44	990
9b	1300	1400	21	1000
10	4200	640	1.8	280

[a] Data are the average of two or more independent experiments. [b] Percentage inhibition at 10 μM concentration.

Table 3. Pharmacokinetic Profile of 10 in Rats[a]

Intravenous (10 mg/kg)	
CL (mL/min kg)	24
V_d (L/kg)	9.5
$t_{1/2}$ (h)	4.6
Intravenous (2 mg/kg)	
C_{brain} at 1.5 h (ng/g)	75
C_{plasma} at 1.5 h (ng/mL)	71
b/p ratio at 1.5 h	1.0
Oral (10 mg/kg)	
T_{max} (h)	0.5
C_{max} (ng/mL)	18
AUC (ng/mL h)	51
F (%)	1

[a] Data are the average of three animals.

of 7.9 in inhibition of α-MSH-stimulated cAMP accumulation (Figure 4).

Selectivity at the Melanocortin Subtype Receptors. Compounds **8**−**10** were profiled for selectivity in binding affinity at the human melanocortin receptor subtypes. These competition binding assays were performed on the HEK 293 cells stably transfected with the human MC1, MC3, and MC5 receptors, using [^{125}I]-NDP-MSH as the radiolabeled ligand. The tested compounds displayed good selectivity of MC4R over other receptor subtypes. For example, compound **10** exhibited K_i values of 4200, 640, 1.8, and 280 nM, respectively, at the human MC1R, MC3R, MC4R, and MC5R (>130-fold selectivity). The results are summarized in Table 2.

Pharmacokinetic Property of Compound 10. The pharmacokinetic profile of **10** was assessed in rats (N = 3). This compound exhibited limited plasma exposure when given orally (1% at 10 mg/kg dose). This was most likely caused by poor intestinal absorption,[28] which could be associated with the high hydrophilicity of the molecule (measured log D value of 1.1 at pH 7.4). After an intravenous administration at 10 mg/kg, the clearance of **10** was 24 mL/min kg, the volume distribution was 9.5 L/kg, and the half-life was 4.6 h in this species. Moreover, **10** penetrated to the brain. Thus, when given intravenously at 2 mg/kg dose, the brain and plasma concentrations of **10** were respectively 75 ng/g and 71 ng/mL at the 1.5 h time point (Table 3).

Discussion

Structural Information of Melanotropin Peptides. All the melanotropin peptides, α-, β-, and γ-MSH and ACTH, possess a HFRW motif, which has been proven to be crucial for the activation of the melanocortin receptors.[13,29] One common feature for potent synthetic melanocortin agonists is the replacement of the L-Phe^7 of α-MSH with its D-isomer. NDP-MSH is one of the most widely used nonselective melanocortin agonists, and it has high binding affinity (K_i = 1.32 nM) and agonist potency (EC_{50} = 0.27 nM) at the human MC4R. SHU9119 is a very close analogue of the cyclic peptide superagonist MT-II (Figure 2). However, SHU9119 is a potent antagonist (IC_{50} = 1.8 nM, pA_2 = 9.3) at the human MC4R.[30] Chemically, the only difference between these cyclic peptides is the D-(2′)Nal residue of SHU9119 and the D-Phe of MT-II.

An alanine-scan of NDP-MSH has revealed that D-Phe^7 is the most important residue of this peptide in both binding and function, followed by Arg^8. Thus, D-alanine replacement of D-Phe^7 results in an over 1100-fold reduction in binding (K_i = 1.6 μM) and function (EC_{50} = 320 nM). Norleucine replacement of Arg^8 gives the peptide with a 200-fold decrease in binding affinity (K_i = 270 nM) and about 55-fold loss in agonist potency (EC_{50} = 15 nM). Alanine replacement of the Trp^9 residue also results in a 100-fold decrease in binding (K_i = 130 nM), but much less reduction in function (EC_{50} = 3.45 nM), and this mutant can be rescued by a phenylalanine.[31]

Mutagenesis of the Melanocortin-4 Receptor and NDP-MSH Peptide. Site-directed mutagenesis studies of the mouse and human melanocortin receptors have established several key residues that are involved in the binding and function of α-MSH and other peptide ligands such as NDP-MSH and MT-II.[31,32] In parallel to the poor interaction of the (D-Phe)7(D-Ala) NDP-MSH mutant with the wild-type human MC4R, NDP-MSH is unable to bind the His264Ala mutant of the human MC4R and exhibited low agonist potency (almost 500-fold increase in EC_{50} value).[32] The binding affinity of

NDP-MSH to the Phe261Ala human MC4R mutant also decreases over 5-fold. In the mouse MC4R, the binding affinity of NDP-MSH to the Phe253Ser mutant is reduced over 10-fold (Phe253 of the mouse MC4R is equivalent to Phe261 of the human MC4R). More interestingly, NDP-MSH can only partially activate this mutant (i.e. acts as a partial agonist).[32] Because of the good match between the magnitudes of reduction in binding and function for the (D-Phe)7(D-Ala) NDP-MSH and the His264Ala human MC4R mutants, it has been suggested that the phenyl group of the NDP-MSH Phe^7 residue mainly interacts with the His264 of the human MC4R. Since Phe261 is located very close to His264 (one loop below in TM-6), it also has a direct or indirect effect on the NDP-MSH binding and function.[32]

One interesting mutant of the mouse MC4 receptor is the Phe254Ser, at which the MC3/MC4 receptor antagonist SHU9119 acts like a partial agonist with an EC_{50} value of 0.83 nM. The Phe254 residue (equal to the Phe262 of the human MC4R) is located very closely to the Phe253 of the mouse MC4R. Data from mutagenesis suggest that a π-rich lipophilic pocket formed by Phe261, Phe262, His264, and other lipophilic amino acids in TM-5 and TM-6 of the human MC4R may host the Phe^7 residue of the melanocortin peptides during their interactions.[31,32]

Three acidic residues, Glu100, Asp122, and Asp126, toward the extracellular surface of TM-2 and TM-3 of the human MC4R are believed to form a negatively charged cage and interact cooperatively with the positively charged guanidinyl moiety of the NDP-MSH Arg^8 residue.[32] The affinity of NDP-MSH binding to the Asp122Ala mutant decreased, albeit only 6-fold, from the wild-type receptor (K_i = 1.54 nM). However, NDP-MSH is unable to bind to either Asp126Ala or Asp126Asn mutant. In addition, the Asp126 mutants respond weakly to NDP-MSH in cAMP stimulation, although the Asp126Asn mutant can be activated by NDP-MSH at a much higher concentration (EC_{50} = 342 vs 0.27 nM at the wild-type). In comparison, Asp122Ala mutation had small effect on NDP-MSH-induced activation (EC_{50} = 1.67 nM). These results suggest that the basic residue of NDP-MSH interacts with both Asp122 and Asp126, but mainly the latter; or the loss of the Asp122 function can be compensated by the Asp126 residue, but not vice versa. The involvement of the Arg^8 residue of NDP-MSH in this interaction is evidenced by the peptide mutant Nle^8[NDP-MSH], which binds to the human MC4R with a much lower affinity (K_i = 264 nM). A double-mutation study has further confirmed the interaction between the Asp122 of the human MC4R and the Arg^8 of NDP-MSH. Thus, the binding affinity of Nle^8[NDP-MSH] at the Asp122Ala mutant is exactly the same as that at the wild-type receptor (K_i = 273 nM, vs 264 nM).

Key Interactions of Compound 4 with the MC4 Receptor. The first reported potent and selective small molecule MC4R agonist **4** was developed from the initial leads **3a,b** (Figure 2),[23] which were in turn designed by replacing the Arg-Trp residues of the His-Phe-Arg-Trp motif with a privileged structure for GPCRs.[24] **3a**, which has a D-2′-naphthylalanine moiety, possesses an IC_{50} value of 108 nM, but little agonist efficacy at the human MC4R. However, **3b**, a D-phenylanaline derivative, displays full efficacy and moderate agonist potency (IA = 97%, EC_{50} = 500 nM, IC_{50} value is not reported). These results are parallel to that observed in peptide ligands. For example, the only difference between the agonist MT-II and the antagonist SHU9119 is a D-Phe residue in MT-II and a D-2′-Nal moiety in SHU9119.[30] Increased potency by introducing a 4-chlorine on the Phe^7 residue of α-MSH analogues has also been observed.[33] Apparently the His residue of **3b** is optimized to the *R*-Tic moiety of **4** for better binding and functional activity (Figure 2). These data strongly suggest that the D-4-chlorophenylanaline of **4** is an equivalent of the Phe^7 residue of the melanotropin peptides. Computational superposition of MT-II and **4** indicates the 4-chlorophenyl group of **4** overlays with D-Phe of MT-II, and the Tic group of **4** sits between His and Trp residues of MT-II.[23] The role of the triazole moiety of **4**, however, is not clear based on this model. In a similar study, superposition of the solution structure of a constrained melanocortin peptide analogue and **4** results in a different orientation,[34] in which, the Tic group takes both Arg and Trp functionalities. Since the triazole of **4** does not mimic any of the HFRW residues of the melanotropin peptide, the authors speculated that this moiety binds in a lipophilic pocket in order to explain the large difference in binding affinity between **5a** and **5b**.[30]

One striking data set is from the small molecule MC4R agonists of the tetrazoles **5a**−**c** (Figure 3). While the *N*-methylated tetrazole **5b** is a potent agonist (EC_{50} = 0.6 nM, IA = 103%) with high binding affinity (IC_{50} = 0.3 nM), the parent tetrazole **5a** exhibited poor binding (IC_{50} = 238 nM) and poor agonist potency (EC_{50} = 771 nM, IA = 79%). In addition, the regioisomeric tetrazole **5c** (IC_{50} = 1.2 nM, EC_{50} = 9.6 nM, IA = 87%) is also a potent MC4R agonist. The poor interaction of **5a** with the MC4R is most likely associated with the acidic nature of the tetrazole. We believe that these results imply that the triazole of **4** or the *N*-methyltetrazole of **5b** or **5c** may interact with acidic residues of the MC4 receptor by partially mimicking, through hydrogen-bonding, the Arg^8 function of the melanotropin peptides. Interestingly, the basicity of the guanidine moiety of the Arg^8 residue of melanotropin peptides seems to be nonessential for interactions with the receptor; thus, an acylguanidine analogue retains the agonist activity of the corresponding guanidine compound.[35]

Three-Dimensional Model of the Human MC4 Receptor. The homology model of the human MC4R based on the crystal rhodopsin template has been built by several different groups.[36] From these three-dimensional structure models, several key residues, which line a putative binding pocket, are identified between TM-3, TM-6, and TM-7 of the transmembrane domains. These amino acids include Phe261, Phe262, and His264 of the TM-6 and Asp122 and Asp126 of the TM-3 (Figure 5).

Bondensgaard and co-workers suggest that the TM-6 lipophilic cavity hosts the privileged structure part of the MC4R agonist **4** on the basis of their computational model.[35b] Alternatively, from the experimental results discussed above, we believe that the 4-chlorophenyl group of **4** might interact with these π-rich residues of

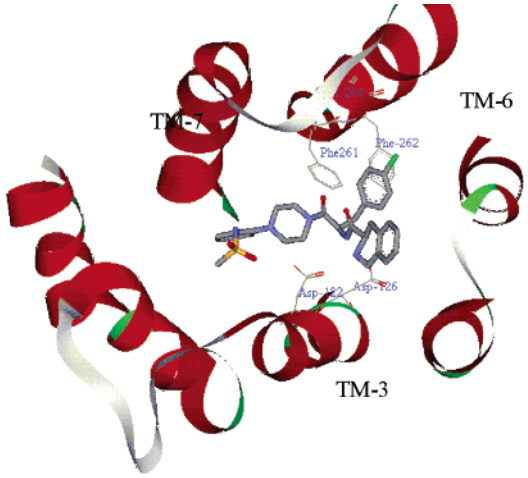


Figure 5. Illustration of small molecule ligand **6b** binding in the putative binding pocket of human MC4 receptor.

TM-6, which is considered to be the primary helix, in addition to TM-3, for GPCRs' activation.[37] The aromatic group of the phenylalanine of **3a** and **3b** is responsible for switching between agonist and antagonist activity. The lipophilic cyclohexyl group of **4**, which is part of the privileged structure, could reside in an area close to TM-7, which has several hydrophobic residues, such as Phe284, Leu288, Ile289, Ile291, and Met292, facing the putative binding pocket. Most importantly, the triazole of **4** now sits very closely to the Asp122 residue of TM-3. Figure 5 illustrates the orientation and location of **6b** in the putative binding pocket of the human MC4R. With one loop separation, the Asp122 residue directly points to the binding pocket, while Asp126 faces TM-5. Asp126 may interact with the basic nitrogen of the Tic group of **4**, which functions as both Arg and His residues of the HFRW motif. The triazole of **4** mainly interacts with the Asp122 residue through hydrogen-bonding.

Small Molecule MC4R Agonist 4 at the Mutant Receptor. Mutagenesis results from our lab have demonstrated that several key residues such as Asp122 and Phe261 of the human MC4R impact on the binding and function of the small molecule MC4R agonist **4**.[27] Mutation of Asp126 to a nonacidic residue abolishes the radiolabeled NDP-MSH binding to the mutant receptor[31,32] and greatly diminishes the cAMP stimulation by melanotropin peptides as well as **4**. Asp122Ala and Asp122Asn mutants, however, are still fully functioning receptors. While the binding affinity of NDP-MSH at the Asp122Ala mutant only decreases 6-fold, **4** has a K_i value of 646 nM at this mutant, a 65-fold reduction from the wild-type. The EC_{50} value of **4** is also increased over 30-fold (EC_{50} = 49 nM, IA = 78%). These results indicate a major involvement of the Asp122 residue in the interaction with small molecule **4**. In addition, the binding affinities of **4** at the Phe261Ala and Phe284Ala mutants are reduced by 48- and 28-fold, respectively.

Rational Design of Piperazinebenzylamines as MC4R Antagonists. Results from mutagenesis, SAR of peptide and small molecule MC4R agonists, and receptor modeling point to a possible interaction between the Asp122 of the human MC4R and the triazole motif of **4** or **6a**. This hypothesis prompted us to synthesize the benzylamine **7** in which the nonbasic triazole of **6a** was replaced with a basic amino group to enhance its possible interaction with an acidic residue of the receptor from hydrogen-bonding to a charge−charge attraction. Not surprisingly, **7** has a K_i value of 380 nM,[38] which is very comparable to the triazole **6** (K_i = 270 nM). From the receptor model, several lipophilic residues, such as Thr101 and Ile104 of TM-2, and Ile125, Ile129, Leu133, and Leu134 of TM-3 could be found near the Asp122 and Asp126 residues. Thus, in addition to hydrogen bonding with Asp122, the triazole of **6a** may also contribute binding energy through lipophilic interaction with these residues. In addition, an empty cavity between TM-3 and TM-4 could be identified from the receptor model (Figure 5). This cavity was lined by several lipophilic residues such as Leu133 and Leu134 of TM-3 and Phe184 of TM-4. On the basis of this observation, we then introduced a lipophilic side chain on the benzylamine **7**. One of these analogues, the 2-(2-thienyl)ethylamine **8a**, exhibits a K_i value of 10.8 nM, a 35-fold increase over **7**. Interestingly, the agonist potency of **8a** only increases by 3-fold (EC_{50} = 290 nM).

After successfully improving the binding affinity of compounds **8**, we then focused on the *R*-Tic group, since this moiety could be important for receptor activation. A possible interaction of the basic nitrogen of the Tic group with the acidic residue of Asp126 on TM-3 was predicted from a preliminary receptor modeling study (Figure 5). On the basis of mutagenesis studies, Asp126 is very important residue for receptor function. To test this hypothesis of the Tic interaction, a glycine analogue **9a**, in which the possible lipophilic interaction of **8a** is eliminated, was synthesized. **9a** has a K_i value of 44 nM, which is only about a 4-fold decrease from **8a**. Like **8a**, compound **9a** also possesses full agonist efficacy (EC_{50} = 580 nM, intrinsic activity = 102%). Very interestingly, by changing the amino functionality of the glycine moiety of **9a**, the agonist efficacy of its analogues is greatly reduced. Thus, the β-alanine analogue **9b** displays slightly increased affinity (K_i = 20.5 nM) but loses most efficacy (cAMP stimulation 14% of α-MSH). Similarly, replacing the glycine of **9a** with a methoxyacetyl group (**9c**) results in a slight reduction in affinity (K_i = 69.5 nM), but much lower efficacy (IA = 16%).

While the Asp122 and Asp126 residues of the human MC4R may function cooperatively, mutagenesis studies suggest that Asp126 is a more important residue than Asp122 in receptor activation by agonists. This could be used to explain why the benzylamine **8a** (K_i = 10.8 nM, EC_{50} = 290 nM) is a weak agonist and the diamine **8b** (K_i = 6.4 nM, EC_{50} = 4.7 nM) is a potent one, while both possesses similar binding affinities. The basic nitrogen of benzylamine in **8a** probably mainly contacts Asp122; however, the second nitrogen of **8b** might interact directly with the Asp126 residue.

Next, we turned our attention to the 4-chlorophenyl group of **9b**. We believe this aromatic ring is an equivalent of the Phe[7] residue of the melanotropin peptides and thus interacts with His264/Phe261 and the adjacent aromatic/lipophilic residues of the human MC4R. The involvement of the Phe261 residue in binding of **4** is evidenced by an almost 50-fold decrease in affinity of **4** binding to the Phe261Ala mutant (K_i = 479 nM).[27] Since the agonist activity of MT-II and compound **3b** can be "switched off" simply by replacing

the phenyl ring with a more lipophilic 2′-naphthyl group, we hypothesized that a similar change could switches off any agonist efficacy of compounds **9b**. Indeed, replacing the 4-chlorophenyl ring of **9b** with a more lipophilic 2,4-dichlorophenyl group (**10**) not only abolishes its intrinsic activity but also increases the binding affinity over 10-fold. Thus, compound **10** has a K_i value of 1.8 nM in binding affinity, and it does not stimulate significant cAMP release mediated by the human MC4 receptor at 10 μM concentration (~3% of the α-MSH level). We also proved that **10** is a competitive antagonist in inhibiting α-MSH-stimulated cAMP production by the Schild plot analysis ($pA_2 = 7.9$, Figure 4). Like **4**, compound **10** is also very selective over the other melanocortin subtypes. Thus, **10** has K_i values of 4200, 640, and 280 nM on the human MC1, MC3, and MC5 receptors, respectively (Table 2). **10** also inhibits the radiolabeled AgRP(87−132) binding to the human MC4R with high potency (K_i = 1.5 nM).

The pharmacokinetic profile of **10** was assessed in rats. After intravenous administration (10 mg/kg), it shows a good half-life of 4.6 h in this species. Most importantly, **10** penetrated to the brain with a brain/plasma (b/p) ratio of about 1 at 1.5 h time point. In human liver microsomes in vitro, **10** exhibited a low intrinsic clearance (CL_{int} = 23 mL/min kg).[39]

Conclusion

We have successfully designed and synthesized a potent and selective antagonist, **10**, of the human MC4 receptor, based on information derived from the receptor structure, mutagenesis, and the structure−activity relationships of peptide ligands and small molecule agonists. The recognition of an acidic residue of the receptor that may be involved in the interaction with small molecule agonists prompted us to introduce a basic moiety into the initial molecule **6** to maximize its binding energy. We were also able to identify a site that may involve receptor activation from the results of peptide agonists and antagonists and applied this information to modify the piperazinebenzylamine into a potent MC4R antagonist. Thus, compound **10** possesses a K_i value of 1.8 nM in binding affinity and a pA_2 value of 7.9 in competitive inhibition of α-MSH-stimulated cAMP production. This potent MC4R antagonist **10** is very selective over the other melanocortin receptor subtypes. It possesses a good half-life in rats, and most importantly, it is also able to penetrate into the brain. The effects of **10** and its analogues on the mutant receptors of Asp122 and other residues lining the binding pocket will be reported elsewhere.

Experimental Section

Chemistry. General Methods. NMR spectra were recorded on a Varian 300 MHz spectrometer with TMS as an internal standard and $CDCl_3$ as the solvent. Chemical shifts are reported in parts per million (δ), and signals are expressed as s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or br (broad). High-resolution mass spectra were measured at the Scripps Center for Mass Spectrometry using MALDI−FTMS. Purity measurements were performed on an HP Agilent 1100 HPLC-MS (detection at 254 nm).

4-{(*2R*)-[(*3R*)-1,2,3,4-Tetrahydroisoquinolinylcarboxamido]-3-(4-chlorophenyl)propionyl}-1-[2-(aminomethyl)phenyl]piperazine (7). A mixture of 2-fluorobenzaldehyde (8.54 mL, 80.54 mmol), 1-(*tert*-butoxycarbonyl)-piperazine (15 g, 80.54 mmol), and potassium carbonate (16.75 g. 121.16 mmol) in DMF (81 mL) was heated at 150 °C for 8 h with constant stirring. The reaction mixture was cooled to room temperature, diluted with ethyl acetate (200 mL), and washed with water (3 × 150 mL) and saturated NaCl solution (150 mL). The organic layer was dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The yellow oil solidified under vacuum overnight, giving a bright yellow solid. The solid was washed with hexanes (3 × 100 mL) to removed impurities, collected, and dried under high vacuum. 2-[4-(*tert*-Butoxycarbonyl)-piperazin-1-yl]benzaldehyde was obtained as a bright yellow solid (17.5 g, 75% yield).

2-[4-(*tert*-Butoxycarbonyl)piperazin-1-yl]benzaldehyde (304.3 mg, 1 mmol) was dissolved in a mixture of methylene chloride/trifluoroacetic acid (2 mL/2 mL) and was stirred vigorously for 30 min at room temperature. Solvents were evaporated, and the residue was dissolved in methylene chloride (5 mL). Diisopropylethylamine (3 mL) was added and evaporation under vacuum gave the free amine intermediate, which (190 mg, 1 mmol), dissolved in DMF (2 mL), was added to a mixture of Boc-D-Tic-D-(4-Cl)Phe-OH [24g] (459 mg, 1 mmol) and HBTU (457 mg, 1.2 mmol), previously stirred in DMF (4 mL) for 30 min at 40 °C. The mixture was stirred at 40 °C for 6 additional hours. Water (5 mL) was added, and the product was extracted with diethyl ether and purified on silica (hexanes/ethyl acetate 1:1) to yield 4-{(*2R*)-[(*3R*)-1,2,3,4-tetrahydroisoquinolinylcarboxamido]-3-(4-chlorophenyl)propionyl}-1-[2-formylphenyl]piperazine (415 mg, 66%).

A solution of the above compound (30 mg, 0.048 mmol) and ammonia/methanol (7 M, 0.023 mL, 0.16 mmol) in methanol (0.5 mL) was stirred for 24 h. Sodium borohydride (3 mg, 0.08 mmol) was added, and the mixture was stirred overnight. The mixture was then diluted with ethyl acetate (5 mL), washed with saturated aqueous sodium bicarbonate (2 mL), dried over sodium sulfate, and concentrated. To the residue were added dichloromethane (0.5 mL) and trifluoroacetic acid (0.5 mL), and stirring was continued for 15 min. The solution was concentrated and then purified by preparative HPLC to give the title compound **7** (TFA salt) as a pale yellow oil (12 mg, 33%). An analytical sample of the free base of **7** was prepared by dissolving in dichloromethane (1 mL), washing with saturated aqueous sodium bicarbonate (1 mL), drying over magnesium sulfate, and evaporating to give a white solid: purity 99% (HPLC); ^{1}H NMR 7.96 (d, J = 8 Hz, 1H), 7.23−7.34 (m, 4H), 7.09−7.21 (m, 6H), 7.02−7.10 (m, 1H), 6.94−7.02 (m, 1H), 5.14−5.24 (m, 1H), 3.80−4.00 (m, 4H), 3.20−3.65 (m, 4H), 3.18 (d, J = 5 Hz, 1H), 3.13 (d, J = 5 Hz, 1H), 2.92−3.08 (m, 3H), 2.54−2.92 (m, 3H), 2.06−2.35 (m, 1H), 1.23−1.29 (m, 1H), 0.82−0.95 (m, 2H); HRMS *m/z* calcd for $C_{30}H_{35}N_5ClO_2$ $(MH)^+$ 532.2474, found 532.2459.

4-{(*2R*)-[(*3R*)-1,2,3,4-Tetrahydroisoquinolinylcarboxamido]-3-(4-chlorophenyl)propionyl}-1-{2-[2-(2-thienyl)-ethylaminomethyl]phenyl}piperazine (8a). To a solution of 4-{(*2R*)-[(*3R*)-1,2,3,4-tetrahydroisoquinolinylcarboxamido]-3-(4-chlorophenyl)propionyl}-1-[2-formylphenyl]piperazine (25 mg, 0.040 mmol) and 2-(2-thienyl)ethylamine (10 mg, 0.080 mmol) in dichloromethane (0.5 mL) was added sodium triacetoxyborohydride (12 mg, 0.080 mmol), and the mixture was stirred overnight. The mixture was then quenched with saturated aqueous sodium bicarbonate (0.5 mL) and separated, and the aqueous layer was extracted with ethyl acetate (1 mL). The combined organics were dried over sodium sulfate and concentrated. To the residue were added dichloromethane (0.5 mL) and trifluoroacetic acid (0.3 mL), and stirring was continued for 30 min. The solution was concentrated then purified by preparative HPLC to give the title product **8a** (TFA salt) as a pale yellow oil (21 mg, 60%). An analytical sample of the free base of **8a** was prepared by dissolving in dichloromethane (1 mL), washing with saturated aqueous sodium bicarbonate (1 mL), drying (magnesium sulfate), and evaporating to give a white solid: purity 95% (HPLC); ^{1}H NMR 7.95 (d, J = 12 Hz, 1H), 7.68−7.72 (m, 1H), 7.51−7.56 (m, 1H), 7.25−7.30 (m, 2H), 7.04−7.23 (m, 7H), 6.89−6.95 (m, 2H), 6.78−6.81 (m, 1H), 5.13−5.22 (m, 1H), 4.16−4.27 (m, 2H), 3.98

(d, J = 6 Hz, 1H); 3.82 (s, 2H), 3.62−3.76 (m, 1H), 3.54 (d, J = 6 Hz, 1H), 3.58 (d, J = 6 Hz, 1H), 3.24−3.52 (m, 2H), 3.18 (d, J = 5 Hz, 1H), 3.15 (d, J = 5 Hz, 1H), 2.95−3.15 (m, 2H), 2.95 (d, J = 6 Hz, 1H), 2.88 (t, J = 6 Hz, 2H), 2.53−2.82 (m, 2H), 2.15−2.25 (m, 1H), 1.59−1.76 (m, 1H), 1.23−1.49 (m, 2H), 0.83−0.98 (m, 1H); HRMS *m/z* calcd for $C_{36}H_{41}N_5ClO_2S$ $(MH)^+$ 642.2664, found 642.2651.

4-{(2*R*)-[2-Aminoacetamido]-3-(4-chlorophenyl)propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]phenyl}-piperazine (9a). To a solution of *N*-Cbz-piperazine (14.2 g, 64.5 mmol) and 2-fluorobenzaldehyde (8.10 g, 65.3 mmol) in dry, degassed DMSO (60 mL) in a pressure tube was added potassium carbonate (12.2 g, 88.3 mmol). The mixture was heated with stirring at 120 °C for 19 h. The mixture was cooled, diluted with ethyl acetate (200 mL), and washed with saturated aqueous ammonium chloride (100 mL). The aqueous layer was extracted with ethyl acetate (100 mL), and the combined organics were dried over sodium sulfate, concentrated in vacuo, and purified by flash column chromatography (10−20% ethyl acetate/dichloromethane) to give 2-(4-Cbz-piperazine)benzaldehyde **11** as a viscous yellow oil (11.0 g, 53%): MS *m/z* 325.0 $(MH)^+$.

Sodium triacetoxyborohydride (4.50 g, 21.2 mmol) was added in portions to a solution of 2-(4-Cbz-piperazine)benzaldehyde (**11**, 4.93 g, 15.2 mmol) and 2-(2-thienyl)ethylamine (2.04 g, 16.0 mmol) in dry dichloromethane (60 mL) over 5 min. The mixture was stirred for 16 h and then was quenched with aqueous saturated sodium bicarbonate (30 mL). The mixture was separated, and the aqueous layer was extracted with dichloromethane (2 × 30 mL). The combined organics were washed with brine (60 mL), dried over magnesium sulfate, and concentrated to give the crude amine (6.79 g). The amine was immediately dissolved in dichloromethane (30 mL), and di-*tert*-butyl dicarbonate (3.49 g, 16.0 mmol) was added. The solution was stirred for 6 h and then diluted with dichloromethane (100 mL), washed with saturated sodium bicarbonate (50 mL) and brine (50 mL), dried over magnesium sulfate, and concentrated. The crude was purified by flash column chromatography (25% ethyl acetate/hexane) to give *N*-Boc-*N*-[2-(2-thienyl)ethyl]-2-(4-Cbz-piperazine)benzylamine **12** as a viscous, pale yellow oil (6.60 g, 82% over two steps): MS *m/z* 536.1 $(MH)^+$.

A mixture of *N*-Boc-*N*-[2-(2-thienyl)ethyl]-2-(4-Cbz-piperazine)benzylamine(**12**, 6.30 g, 11.8 mmol) and 10% Pd/C (650 mg) in ammonia/methanol (7 M, 80 mL) was hydrogenated in a Parr apparatus at 40 psi for 1 h. A second batch of catalyst (650 mg) was added, and the mixture was hydrogenated for 4 h. A third batch of catalyst (650 mg) was added, and the mixture was hydrogenated for 18 h and then filtered through Celite, concentrated in vacuo, and purified by flash column chromatography. The remaining starting material was eluted first (50% ethyl acetate/hexane) followed by *N*-Boc-N-[2-(2-thienyl)ethyl]-2-piperazinebenzylamine as a viscous, pale yellow oil (10% methanol/dichloromethane) (1.65 g, 35%): MS *m/z* 402.0 $(MH)^+$.

To a mixture of *N*-Boc-*N*-[2-(2-thienyl)ethyl]-2-piperazinebenzylamine (880 mg, 2.19 mmol) and *N*-Fmoc-D-(4-chlorophenyl)-alanine (1.02 g, 2.41 mmol) in dichloromethane (30 mL) was added HOBt (325 mg, 2.41 mmol), and the mixture was stirred for 20 min. EDC (460 mg, 2.41 mmol) was added, and stirring was continued for 18 more hours. The mixture was then washed with saturated sodium bicarbonate (2 × 15 mL) and brine (15 mL), dried over magnesium sulfate, and concentrated in vacuo. The crude **13a** was filtered through silica gel (10% ethyl acetate/dichloromethane), concentrated, and dissolved in a 1:1 mixture of diethylamine/dichloromethane (20 mL). After stirring for 3 h, the solution was concentrated and the product isolated by flash column chromatography (9:1 ethyl acetate/dichloromethane to 94:5:1 dichloromethane/methanol/triethylamine) to give 4-[(2*R*)-amino-3-(4-chlorophenyl)propionyl]-1-{2-[*N*-Boc-(2-thienyl)ethylaminomethyl]phenyl}piperazine as a white foam (1.11 g, 87% over two steps): MS *m/z* 583.2 $(MH)^+$.

To a mixture of 4-[(2*R*)-amino-3-(4-chlorophenyl)propionyl]-1-{2-[*N*-Boc-(2-thienyl)ethylaminomethyl]phenyl}piperazine (30 mg, 0.052 mmol) and *N*-Boc-glycine (10 mg, 0.057 mmol) in dichloromethane (1 mL) was added HOBt (9.0 mg, 0.057 mmol), and the mixture was stirred for 10 min. EDC (11 mg, 0.057 mmol) was added, and stirring was continued overnight. The mixture was washed with saturated aqueous sodium bicarbonate (1 mL) and separated, and the aqueous layer was extracted with ethyl acetate (1 mL). The combined organics were dried over sodium sulfate and concentrated. To the residue was added 4 M HCl/dioxane (1 mL), and the mixture was stirred for 2 h and then concentrated and purified by preparative HPLC to give the title product **9a** (TFA salt) as a pale yellow oil (26 mg, 65%). An analytical sample of the free base of **9a** was prepared by dissolving in dichloromethane (1 mL), washing with saturated aqueous sodium bicarbonate (1 mL), drying over magnesium sulfate, and evaporating to give a white solid: purity 100% (HPLC); ^{1}H NMR 7.88 (d, J = 9 Hz, 1H), 7.06−7.33 (m, 9H), 6.88−6.93 (m, 2H), 6.79−6.81 (m, 1H), 5.15−5.23 (m, 1H), 3.82 (bs, 2H), 3.61−3.72 (m, 1H), 3.19−3.45 (m, 3H), 3.36 (s, 2H), 2.94−3.09 (m, 4H), 2.88 (t, J = 7 Hz, 2H), 2.69−2.77 (m, 1H), 2.50−2.64 (m, 2H), 1.90−2.20 (m, 2H), 1.22−1.29 (m, 1H); HRMS *m/z* calcd for $C_{28}H_{35}N_5ClO_2S$ $(MH)^+$ 540.2194, found 540.2188.

4-{(2*R*)-[3-Aminopropionylamido]-3-(4-chlorophenyl)-propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]phenyl}-piperazine (9b). This compound was synthesized using a procedure similar to that described for **9a** with *N*-Boc-β-alanine: white solid (100% purity, HPLC); ^{1}H NMR 7.71 (bs, 1H), 7.02−7.36 (m, 7H), 6.86−6.96 (m, 2H), 6.78−6.82 (m, 1H), 5.15−5.21 (m, 1H), 3.80 (bs, 1H), 3.51−3.55 (m, 2H), 3.48 (s, 1H), 2.11−3.47 (m, 18H), 1.17−1.29 (m, 1H), 0.83−0.94 (m, 1H); HRMS *m/z* calcd for $C_{29}H_{37}N_5ClO_2S$ $(MH)^+$ 554.2351, found 554.2350.

4-{(2*R*)-[2-Methoxyacetamido]-3-(4-chlorophenyl)propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]phenyl}-piperazine (9c). This compound was synthesized using a procedure similar to that described for **9a** with 2-methoxyacetic acid: colorless oil (95% purity, HPLC); ^{1}H NMR 7.06−7.34 (m, 7H), 6.87−6.93 (m, 2H), 6.79−6.81 (m, 1H), 5.17−5.25 (m, 1H), 3.90 (d, J = 4.2 Hz, 2H), 3.80 (s, 2H), 3.65−3.94 (m, 3H), 3.42 (s, 3H), 3.15−3.35 (m, 3H), 2.91−3.08 (m, 4H), 2.87 (t, J = 6.0 Hz, 2H); 2.68−2.80 (m, 1H), 2.49−2.65 (m, 2H), 2.02−2.18 (m, 1H), 1.25 (s, 1H); HRMS *m/z* calcd for $C_{29}H_{35}N_4ClO_3S$ $(MH)^+$ 555.2191, found 555.2199.

4-{(2*R*)-[3-Aminopropionylamido]-3-(2,4-dichlorophenyl)propionyl}-1-{2-[(2-thienyl)ethylaminomethyl]phenyl}-piperazine (10). To a mixture of *N*-Boc-*N*-[2-(2-thienyl)ethyl]-2-piperazinebenzylamine (880 mg, 2.19 mmol) and *N*-Fmoc-D-(2,4-dichlorophenyl)alanine (1.02 g, 2.41 mmol) in dichloromethane (30 mL) was added HOBt (325 mg, 2.41 mmol), and the mixture was stirred for 20 min. EDC (460 mg, 2.41 mmol) was added, and stirring was continued for 18 more hours. The mixture was then washed with saturated sodium bicarbonate (2 × 15 mL) and brine (15 mL), dried over magnesium sulfate, and concentrated in vacuo. The crude **13b** was filtered through silica gel (10% ethyl acetate/dichloromethane), concentrated, and dissolved in a 1:1 mixture of diethylamine/dichloromethane (20 mL). After stirring for 3 h, the solution was concentrated and the product isolated by flash column chromatography (9:1 ethyl acetate/dichloromethane to 94:5:1 dichloromethane/methanol/triethylamine) to give 4-[(2*R*)-amino-3-(2,4-dichlorophenyl)propionyl]-1-{2-[*N*-Boc-(2-thienyl)ethylaminomethyl]phenyl}piperazine as a white foam (1.11 g, 87% over two steps): MS *m/z* 583.2 $(MH)^+$.

To a mixture of 4-[(2*R*)-amino-3-(2,4-dichlorophenyl)propionyl]-1-{2-[*N*-Boc-(2-thienyl)ethylaminomethyl]phenyl}piperazine (26 mg, 0.036 mmol) and *N*-Boc-β-alanine (7.0 mg, 0.036 mmol) in dichloromethane (1 mL) was added HOBt (5.0 mg, 0.036 mmol), and the mixture was stirred for 10 min. EDC (7.0 mg, 0.036 mmol) was added, and stirring was continued overnight. The mixture was washed with saturated aqueous sodium bicarbonate (1 mL) and separated, and the aqueous layer was extracted with ethyl acetate (1 mL). The combined

organics were dried over sodium sulfate and concentrated. To the residue was added 4 M HCl/dioxane (1 mL), and the mixture was stirred for 2 h and then concentrated and purified by preparative HPLC to give the title product **10** (TFA salt) as a pale yellow oil (10 mg, 34%). An analytical sample of the free base of **10** was prepared by dissolving in dichloromethane (1 mL), washing with saturated aqueous sodium bicarbonate (1 mL), drying over magnesium sulfate, and evaporating to give the title compound as a white solid: purity 100% (HPLC); ^{1}H NMR 7.63 (d, J = 7 Hz, 1H), 7.41 (s, 1H) 7.07−7.26 (m, 6H), 6.85−6.95 (m, 2H), 6.80 (d, J = 3 Hz, 1H), 5.30−5.39 (m, 2H), 3.80 (bs, 2H), 3.17−3.79 (m, 4H), 2.90−3.17 (m, 5H), 2.83−2.90 (m, 2H), 2.65−2.81 (m, 3H), 2.42−2.65 (m, 2H), 2.15−2.42 (m, 3H), 1.21−1.32 (m, 1H); HRMS *m/z* calcd for $C_{29}H_{36}N_5Cl_2O_2S$ $(MH)^+$ 588.1961, found 588.1980.

Receptor Binding. Receptor binding was performed on HEK293 cells stably expressing the human melanocortin receptors, using [^{125}I]NDP-MSH as the radiolabeled ligand. The cAMP stimulation and inhibition assays were performed in the same cell lines using α-MSH (as the standard in agonist assay). The assay conditions were similar to those previously reported.[27]

The Three-Dimensional Structure of the MC4 Receptor and Small Molecule Docking. The homology 3D structure of the human MC4 receptor has been built by several groups.[36] The receptor model used in this study was provided by Yang and co-workers.[36a] The small molecule with a low-energy conformation, selected from random conformation search results, was initially placed in the transmembrane domain. The MMFF94 force field with implicit solvation model implemented in MOE was used. The receptor was rigid and the ligand was flexible. A Tabu search with default settings in MOE-dock was performed aiming to optimize both spatial contacts and electostatic interactions. It used a stochastic searching algorithm and wrote the resulting docking conformations and their energies to a molecular database file. The resulting docking conformations were ranked by both total energy and ligand energy. The top ranked solutions were visually evaluated, and the one that best explains the mutagenesis study results is illustrated in Figure 5.

Pharmacokinetic Characterization. Compound was dosed as a hydrochloride salt in aqueous solution. The pharmacokinetic profile of **10** was determined in male mice (N = 3/time points at a dose of 10 mg/kg). The dosing solution was prepared in purified water and filtered through a 0.2 μm Nylon filter before administration (2 mL/kg) via the tail vain (iv) or oral gavage (po). The bioanalytical method applied for the measurement of test articles in plasma along with added internal standard consisted of precipitation with 200 μL of acetonitrile from 50 μL of plasma, centrifugation, and recovery the supernatant, which was evaporated in a vacuum and then reconstituted in acetonitrile/water solution before introduction into an LC−MS/MS system for analysis. The lower limit of quantification (LLOQ) for the analytical methods was 5 ng/mL of test article in plasma. All pharmacokinetic parameters were calculated from a noncompartmental model using WinNonlin program version 3.2.

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