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2,3-Diaryl-5-anilino[1,2,4]thiadiazoles as Melanocortin MC4 Receptor Agonists and Their Effects on Feeding Behavior in Rats

Kevin Pan,[†] Malcolm K. Scott, Daniel H. S. Lee,[‡] Louis J. Fitzpatrick, Jeffery J. Crooke, Ralph A. Rivero,[§] Daniel I. Rosenthal, Anil H. Vaidya, Boyu Zhao and Allen B. Reitz*

Drug Discovery Division, Johnson & Johnson Pharmaceutical Research and Development, Welsh and McKean Rds., PO Box 776, Spring House, PA 19477, USA

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Abstract—The melanocortin-4 receptor (MC4) modulates physiological functions such as feeding behavior, nerve regeneration, and drug addiction. Using a high throughput screen based on ^{125}I-NDP-MSH binding to the human MC4 receptor, we discovered 2,3-diaryl-5-anilino[1,2,4]thiadiazoles **3** as potent and selective MC4 receptor agonists. Through SAR development on the three attached aryl rings, we improved the binding affinity from 174 nM to 4.4 nM IC_{50}. When delivered intraperitoneally, compounds **3a**, **3b**, and **3c** induced significant inhibition of food intake in a fasting-induced feeding model in rats. When delivered orally, these compounds lost activity, mainly due to rapid metabolism to inactive imidoylthiourea reduction products.

Introduction

Five melanocortin G-protein receptor subtypes (MC1–MC5) have been cloned and characterized.[1] Melanocyte-stimulating hormone (MSH) and adrenocorticotropic hormone (ACTH), which are produced from the proteolytic cleavage of a 31–36 KD glycosylated protein called pro-opiomelanocortin (POMC), are two natural agonists of MC receptors. α-MSH contains the first 13 N-terminal amino acids of ACTH. Homologous to α-MSH are two additional peptides, β-MSH and γ-MSH, contained in the POMC sequence. All of these natural ligands bind to MC receptors with differing affinities, with the exception of MC2 which is activated by only ACTH.[2] MC receptors are among the very few receptors that have both natural agonists and antagonists. For example, the agouti signaling protein (ASIP) is an antagonist of MC1 and MC4 receptors.[3] Through a homology search of the genes for proteins related to ASIP, another protein, agouti-gene related protein (AGRP), was cloned and found to be an antagonist of MC3 and MC4 receptors.[4]

All five MC receptors exhibit diverse physiological functions mainly through cAMP stimulation and downstream signal transduction. Expressed in melanocytes of the dermis, the MC1 receptor plays a major role in controlling the formation of the pigment melanin and animal coat coloration. The MC2 receptor is highly expressed in the adrenal cortex and adipose tissues, and is believed to be responsible for the ACTH-mediated control of glucocorticoid and mineralcorticoid production. The MC3 receptor is expressed in the CNS, placenta, gut and heart. Although the functions of the MC3 receptor are not well understood, MSH and ACTH peptides are believed to induce sexual behavior in animals via the stimulation of the MC3 receptor at a hypothalamic site. The MC4 receptor is primarily expressed in the brain and plays an important role in regulating feeding behavior.[5] The MC4 receptor is also involved in drug addiction,[6] nerve regeneration,[7,8] and the regulation of the cardiovascular system together with non-melanocortin receptors.

The role of the MC4 receptor in controlling feeding behavior and body weight is well supported by several studies. For example, direct injection of α-MSH or ACTH-(1–24) into the hypothalamus caused a significant reduction of food intake in rats.[9] Similar effects were also observed when the unnatural agonistic peptide

*Corresponding author. Tel.: +1-215-628-5615; fax: +1-215-628-4985; e-mail: areitz@prdus.jnj.com
[†]Present affiliation: Shanghai Hutchinson Pharmaceuticals.
[‡]Present affiliation: Biogen Inc., 14, Cambridge Center, Cambridge, MA.
[§]Present affiliation: GlaxoSmithKline, King of Prussia, Pennsylvania.

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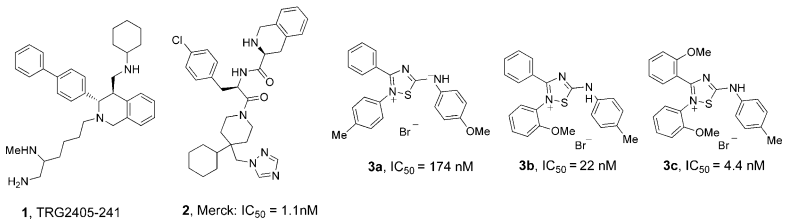


Figure 1.

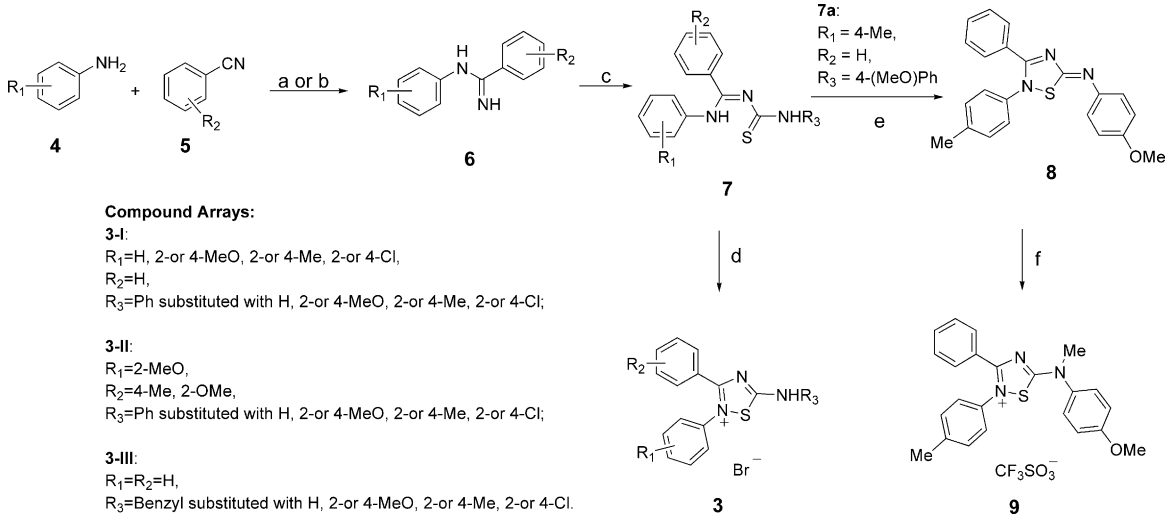
MTII was administered to C57BL/6J mice, *ob*/*ob* mice, A^Y mice, and mice injected with neuropeptide Y.[10] The natural antagonist agouti is overexpressed in the CNS of A^Y agouti mice that developed extreme obesity because of overeating.[11] Intracerebroventricular (icv) administration of the MC4 receptor antagonist SHU9119, a mimic of the MC-4 antagonist agouti, resulted in stimulated nocturnal feeding in C57BL/6J mice.[12] Additionally, icv administration of the MC4 receptor antagonist HS014 also increased food intake in free-feeding rats.[10] Importantly, MC-4 receptor knockout mice developed obesity syndrome resembling some of the characteristic features of the agouti obesity syndrome,[13] and several MC4 receptor mutations were discovered in the human population associated with obesity.[14] Thus, the MC4 receptor is an important target for discovery of drugs to treating obesity, which represents a large and presently poorly treated medical need.[15]

Peptide and peptidomimetic agonists for MC1–MC5 have been reported containing the critical four amino acid pharmacophore His-Phe-Arg-Trp found in MSH.[16–18] NDP-MSH ([Nle4, D-Phe7]-α-MSH) and its radiolabeled analogues have been used extensively to measure the MC receptor binding affinities of nonlabeled compounds and to probe the pharmacology of the MC receptors. Clinical studies of a potent nonselective cyclic heptapeptide agonist at MC receptors, melanotan-II, showed it to be effective in causing erections in men with psychogenic erectile dysfunction.[19] However, MSH-like peptide analogues have limited utility due to their poor bioavailability and difficulties in penetrating the brain–blood barrier. The intense interest in finding small, non-peptide molecules is represented by isoquinoline analogues **1** and **2** (Fig. 1). With low micromolar affinities to the MC1 and MC4 receptors, **1** was reported to reduce dermal inflammation induced by arachidonic acid, food consumption, and body weight.[20] Isoquinoline analogues with a 4,4-disubstituted piperidine moiety, such as **2**, are hMC4 receptor agonists with subnanomolar potency and in vivo erectogenic effects in rodents.[21]

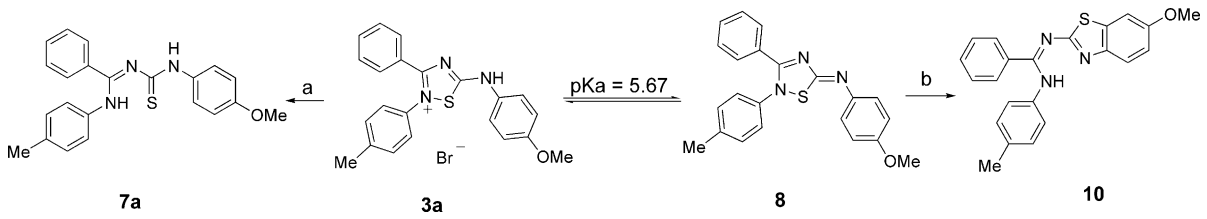
Our interest in the area of selective MC4 agonists prompted us to carry out MC4r high-throughput screening (HTS) of our corporate compound collection, led to the discovery of 2,3-diaryl-5-anilino-[1,2,4]thiadiazolium bromide **3a** as an MC4 receptor agonist. After structure–activity relationship (SAR) studies around the central thiadiazolium scaffold and improving the binding affinity from 174 to 4.4 nM IC_{50}, representative compounds were tested in a fasting-induced rat-feeding model and inhibited food intake.

Synthetic Chemistry

The synthesis of [1,2,4]-thiadiazoles has been reported extensively.[22] In Scheme 1, substituted anilines **4** were reacted with benzonitriles **5** and $AlCl_3$ at an elevated temperature to form *N*-arylbenzamidines **6**.[23] In the presence of a methoxy group, a strong base such as sodium amide was used in the reaction to make **6**.[24] *N*-Arylbenzamidines **6** were reacted with substituted



Scheme 1. (a) $AlCl_3$/neat, 170–220 °C; (b) $NaNH_2$, toluene, reflux; (c) R_3NCS, 55 °C, 1,2-dichloroethane; (d) Br_2, chloroform; (e) *N*-chlorosuccinimide, chloroform; (f) methyl triflate, dichloromethane.



Scheme 2. (a) Dithioerythritol (DTE), dichloromethane; (b) DMSO, 65 °C.

isothiocyanates to form the imidoylthioureas **7**,[25] which were oxidized by bromine to yield thiadiazolium salts **3**. The free bases of **3**, [1,2,4]-thiadiazolines **8**, were prepared by treating **7** with *N*-chlorosuccinimide (NCS) followed by extraction from aqueous sodium bicarbonate. 2,3-Diaryl-5-(*N*-methyl-*N*-arylamino)-[1,2,4]-thiadiazolium triflate **9** was prepared by selective *N*-methylation of compound **8** by methyl triflate. 2-(4-Methylphenyl)-3-phenyl-5-(4-methoxyphenylamino)-[1,2,4]-thiadiazolium bromide **3a** was rapidly reduced by dithioerythritol (DTE) back to the corresponding thiourea (**7a**, Scheme 2).[26] Compound **3a** also underwent a thermal rearrangement to 2-amidinobenzothiazole **10** (biologically inactive at the MC4 receptor), a general reaction that we have studied in some detail.[27]

Biological Evaluation

The affinities of compounds **3**, **8**, and **9** for the MC4 receptor were evaluated in binding assays. Membranes from human Bowes melanoma cells that overexpress the MC4 receptor were immobilized on wheat germ agglutinin-conjugated scintillation proximity assay (SPA) beads, and binding of ^{125}I-NDP-MSH was monitored in the presence and absence of test inhibitors.[28] The data are reported as IC_{50} values in Figures 2 and 3. The in vivo effects of compounds **3a**–**3c** were examined for their ability to inhibit food-intake in fasted rats (Tables 1 and 2).[29] The agonist character of the interaction of compound **3a** (IC_{50} = 174 nM) at the MC4 receptor was confirmed by increased ^{35}S-GTPγS binding.[30] The binding affinity of **3a** to the MC3 receptor was also determined, with an IC_{50} value of 2.15 μM.

Results and Discussions

It was reported that [1,2,4]-thiadiazoles were reduced to imidoylthioureas by H_2S and NH_3.[31] In our hands, **3a** was rapidly reduced to its corresponding precursor imi-

Melanocortin MC-4 IC_{50}s

	R_1 Substituent						
R_3 Sub.	2-MeO	H	4-MeO	2-Me	4-Me	2-Cl	4-Cl
Ph	68	124	106	201	234	482	482
4-(MeO)Ph	48	124	79	217	174	237	424
2-(MeO)Ph	103	76	72	130	159	89	351
4-MePh	22	57	159	243	297	246	463
2-MePh	48	63	146		207	368	460
4-ClPh	91	89	133	347	293	874	636
2-ClPh	46	96	483	234	218	444	392

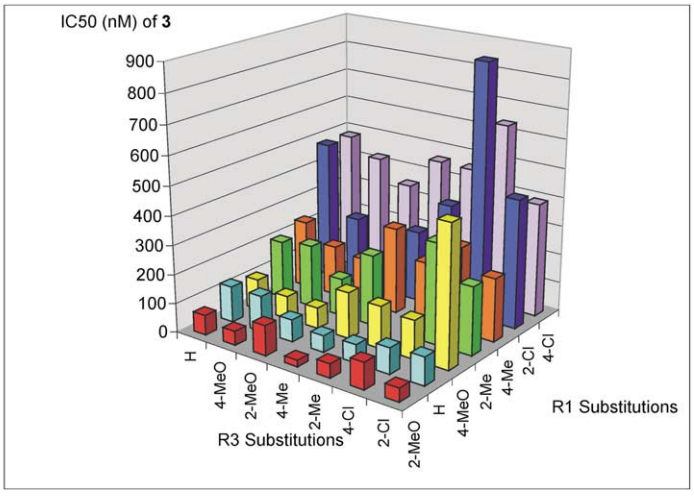


Figure 2. Binding affinities of 2-aryl-3-phenyl-5-anilino[1,2,4]thiadizolium bromides **3** to the MC4 receptor—Part I.

doylthiourea **7a** by DTE (Scheme 2). We had suspected that **3a** might also react with a thiol group on cysteine residues or oxidize two thiol groups to create a disulfide bond, to promote irreversible binding to the receptor. This possibility was discounted after we found that the receptor remained active in binding to ^{125}I-NDP-MSH after **3a** was washed off.

As the first step of SAR exploration, the proton on the 5-anilino nitrogen was replaced by a methyl group (**9**). Compared to **3a**, the activity was essentially eliminated from a 174 nM to 30 μM IC_{50}. This observation strongly suggests that the proton on the anilino nitrogen is required for binding. It is possible that the positive charge on the thiadiazolium ring may also be important in binding. The core tetrapeptide (His-Phe-Arg-Trp) in the natural ligand MSH contains a positive charge on the guanidine moiety. The charged arg residue is thought to play an important role in binding to Glu-94, Asp-121 and Asp-117 in the MC1 receptor.[32] When **3a** was converted to its free base **8**, the apparent activity decreased from a 174 to 833 nM IC_{50}. The pK_a of **3a** was determined to be 5.67.[33]

We then modified aromatic substitution on the 2-phenyl ring (R_1) and the 5-anilino ring (R_3) simultaneously by preparing an array of compounds **3-I** (Fig. 1). In addition to the unsubstituted phenyl ring, methoxy, methyl, and chloro groups were placed at 2- and 4-positions separately. For R_1 substitution, 2-methoxy or no substituent was generally better than the others, and 2- and 4-methyl as well as 2- and 4-chloro substitution was not favored. R_3 substitution was not as discriminating as that at R_1. Electron-rich substitution such as methyl or methoxy groups generally showed better activities. A combination of 2-methoxy on R_1 and 4-methyl on R_3 resulted in **3b** with an IC_{50} value of 22 nM at MC4, an eight-fold improvement of the binding affinity compared to **3a**.

After selecting 2-(2-methoxyphenyl) substitution, we systematically altered the 3-phenyl (R_2) and 5-anilino rings (R_3) to make compounds **3-II** (Fig. 2). When R_2

Melanocortin MC-4 IC_{50}s

	R_2 Substituent		
R_3 Sub.	H	2-MeO	4-Me
Ph	68	174	114
4-(MeO)Ph	48	103	120
2-(MeO)Ph	103	332	130
4-MePh	22	4.4	110
2-MePh	48	99	139
4-ClPh	91	216	566
2-ClPh	46	378	234
3-PyPh	725	672	155

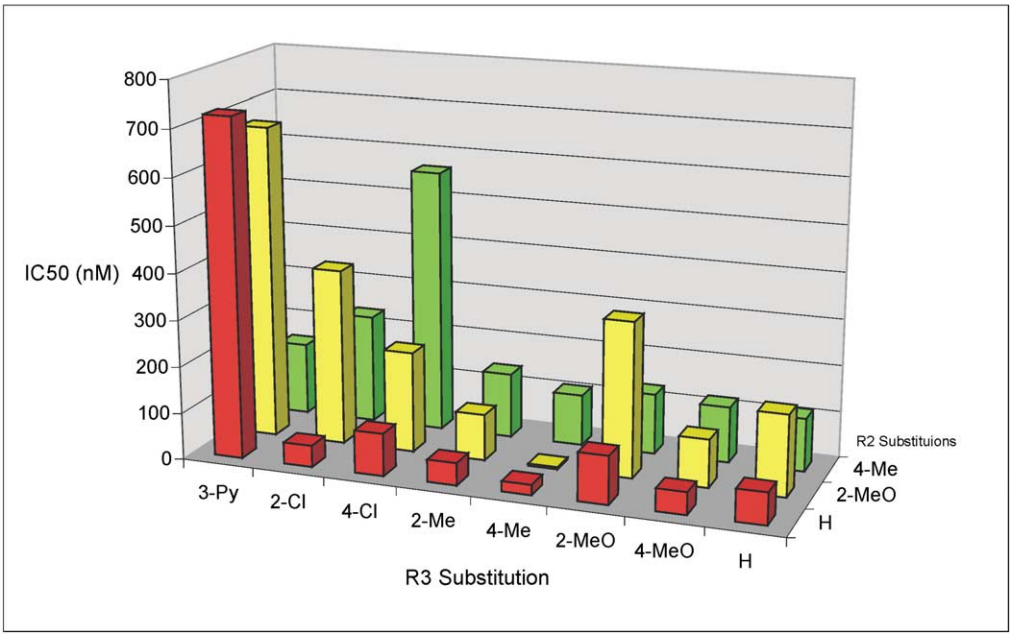


Figure 3. Binding affinities of 2-aryl-3-phenyl-5-anilino[1,2,4]thiadizolium bromides **3** to the MC4 receptor—Part II.

Table 1. Percentage change of food consumption versus baseline day in rats (one-sample *t*-test)

Compd no.	Dose (mg/kg)/route	No. of animals	2 h	4 h	6 h	2–6 h
PEG–200	0/IP[a]	8	0.0	0.0	3.8	6.9
MCT	0/PO[b]	8	11.2	0.0	1.1	−9.0
3a	10/IP	15	−39.0[c]	—	−42.7[c]	−13.8
3a	30/PO	8	1.3	2.8	0.7	0.0
3b	3/IP	8	−37.8[c]	−42.1[c]	−44.4[c]	−50.0[c]
3b	1/IP	8	−33.3[c]	−42.4[c]	−51.3[c]	−68.8[c]
3b	30/PO	8	−10.5	−9.6	9.3	29.7[c]
3b	10/PO	8	2.7	5.4	0.0	−1.9
3b	3/PO	8	−2.4	7.7	13.6	29.1[c]
3c	10/IP	5	−11.1	2.8	−4.2	0.9
3c	3/IP	7	0.0	−4.0	−6.1	−9.9

[a]Intraperitoneal administration.
[b]Oral administration.
[c]*P* value <0.05.

Table 2. Percentage change of food consumption versus drug day (vehicle) in rats (Mann–Whitney *t*-test)

Compd no.	Dose (mg/kg)/route	No. of animals	2 h	4 h	6 h	2–6 h
3a	10/IP[a]	15	−22.1[c]	−12.3	−22.9[c]	−23.3[c]
3a	30/PO[b]	8	−21.2[c]	−11.1	−14.4[c]	−6.2
3b	3/IP	8	−37.8[c]	−37.4[c]	−37.1[c]	−36.2[c]
3b	1/IP	8	−20.6[c]	−45.2[c]	−50.0[c]	−71.8[c]
3b	30/PO	8	−23.6[c]	−12.7[c]	1.2	31.5[c]
3b	10/PO	8	−10.1	−0.9	12.4[c]	39.7[c]
3b	3/PO	8	−9.0	6.8	18.5[c]	52.1[c]
3c	10/IP	5	−11.1	2.8	−4.2	0.9
3c	3/IP	7	0.0	−4.0	−6.1	−9.9

[a]Intraperitoneal administration.
[b]Oral administration.
[c]*P* value <0.05.

was substituted by a 2-methoxy or 4-methyl group, the binding affinities were generally reduced except with a 2-methoxy group on R_2 and a 4-methyl group on R_3 resulting in **3c** with an IC_{50} of 4.4 nM, a 5-fold improvement from **3b**. Indeed, a 4-methyl group was the most optimal substitution on R_3, and electron-deficient 3-pyridyl or chloro substituted phenyl rings were not generally favored.

We also replaced the 5-anilino group of **3** by 5-benzylamino moieties. Compared to their direct anilino analogues, 2,3-diphenyl-5-benzylaminothiadiazolium bromides **3-III** where the benzyl group was optionally substituted with methoxy, methyl, or chloro groups at the 2- or 4-positions were inactive at MC4 (800 to 3000 nM IC_{50}s, data not shown).

Compounds **3b** and **3c** were also determined to be agonists in the functional assays, and were tested along with **3a** in a fasting-induced rat-feeding model. Table 1 includes data based on the change in food consumed between the baseline relative to the drug day in individual rats where each rat was used as its own control. A one-sample *t*-test analysis was used to determine whether the change in food consumed was statistically significant. Table 2 contains data based on the change in food consumed from vehicle in separate rats on the drug day. When compounds were delivered by an intraperitoneal route of administration, except for **3c**, they showed significant effects in reducing food intake in animals. The inhibitory effects on the cumulative food intake after 2, 4, and 6 h periods increased with the time after compound administration, and effects on the average food intake for the 2–6 h time block were generally greater than that at the 6 h period. This observation indicates that maximum effectiveness appeared at least 2 h after administration. When compound **3a** and **3b** were administered ip at 10 mg/kg, they were equally effective on the inhibition of the cumulative food intake, and **3b** appeared to be more active than **3a** when comparing average food intake for the 2–6 h block. It is interesting to see that compound **3c**, which is more active in binding, did not show significant effects on food intake, suggesting that **3c** may have some other general properties which prevent in vivo activity.

When **3a**–**3c** were administered orally, they did not demonstrate significant inhibition on food intake. The in vitro metabolism of **3a** was evaluated using human and rat hepatic S9 fractions (60 min incubation). In this study, only 5% of **3a** remained in the human S9 fraction, and 9% in the rat S9 fraction. The major metabolite was imidoylthiourea **7a** (59%, human S9; 50%, rat S9). Compound **7a** was found to be inactive in the receptor binding assay (>10000 nM IC_{50}) and was also inactive in the rat-feeding model (ip dosing).

Conclusions

The MC4 receptor agonistic activity of the title compounds provides an additional chemical series of small, non-peptidic molecules with activity in a feeding model in rodents. We demonstrated that the 2,3-diaryl-5-anilino-[1,2,4]-thiadiazoles can bind to the MC-4 receptor with low nanomolar affinity. Importantly, these compounds inhibit food intake in rats when administered intraperitoneally. Although the rapid metabolism of these compounds precludes their immediate use as oral drugs for the treatment of obesity, they have provided useful information for further SAR development and research.

Experimental

General. ^{1}H NMR spectra were obtained on a 300-MHz Bruker Avance 300 NMR spectrometer with Me_4Si as an internal standard. The mass spectrum of each compound was generated from a MicroMass Platform LC electrospray mass spectrometer or chemical ionization on a Hewlett-Packard 5989A mass spectrometer. Chromatographic separations were performed using a Biotage Flash 40 flash chromatography system. Most reagents and solvents were purchased and used without further purification.

***N*-(4-Methylphenyl)benzamidine (6a).** Benzonitrile (10.0 g, 97.0 mmol) was slowly added to dry $AlCl_3$ (14.23 g, 106.7 mmol) in a round-bottom-flask. After the hot flask was cooled to rt, 4-toluidine (10.4 g, 97.0 mmol)

was added to the flask, and the reaction was heated at 160 °C for 45 min. The hot mixture was poured into a solution of 3.2 mL of concentrated HCl solution in 240 mL of mixed water and ice. Activated carbon was added and the reaction mixture was filtered through a Celite pad after stirring for 20 min. The filtrate was mixed with a solution of 32 g NaOH solids dissolved in 200 mL water. The aqueous layer was extracted with 3×150 mL of chloroform. The combined organic layer was dried over anhydrous $MgSO_4$, and evaporated. The resulting solid was washed with hexane and dried over the vacuum to yield the product **6a** as a pale white solid (11.4 g, 56%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.88 (d, 2H), 7.45 (m, 3H), 7.19 (d, 2H), 6.92(d, 2H), 4.85 (s, 2H), 2.35 (s, 3H); MS (CI, MH^+) $m/e = 211$.

Compound **6b** was prepared in a similar fashion to give a pale white solid (34%). ^{1}H NMR (300MHz, $CDCl_3$) δ 8.27 (s, 1H), 7.39 (t, 1H), 6.94–7.11(m, 7H), 5.55 (s, 1H), 3.90 (s, 3H), 3.82 (s, 3H); MS (CI, MH^+) $m/e = 257$.

1-(4-Methoxyphenyl)-3-[*N*-(4-methylphenyl)benzimidoyl]-2-thiourea (7a). A mixture of *N*-(4-methylphenyl)benzamidine **6a** (2.10 g, 10.0 mmol) and 4-methoxyphenyl isothiocyanate (1.39 mL, 10.0 mmol) in 30 mL of anhydrous 1,2-dichloroethane was heated at 55 °C for 16 h. The reaction mixture was cooled and the solvent was evaporated. The resulting residue was purified by flash column chromatography with a mobile phase of 25% hexane in dichloromethane. The combined fractions were evaporated; and the resulting solid was dried over vacuum to yield the product **7a** as yellow solid (1.54 g, 41%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.19 (s, 1H), 8.10 (s, 1H), 7.50 (d, 2H), 7.27–7.39 (m, 5H), 6.96 (t, 4H), 6.64 (d, 2H), 3.83 (s, 3H), 2.24 (s, 3H); MS (ES, MH^+) $m/e = 376.25$.

Compounds **7b** and **7c** were prepared in a similar fashion to give yellow solids.

7b (79% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 13.90 (s, 1H), 8.18 (s, 1H), 7.94 (d, 1H), 7.26–7.38 (m, 7H), 7.19 (d, 1H), 6.93 (t, 1H), 6.75 (t, 2H), 6.67 (d, 1H), 3.66 (s, 3H), 2.36 (s, 3H); MS (ES, MH^+) $m/e = 376.29$.

7c (61% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 14.37 (s, 1H), 8.15 (s, 1H), 7.65 (d, 2H), 7.33 (t, 1H), 7.12–7.19 (m, 3H), 6.96 (t, 1H), 6.83 (t, 2H), 6.77 (d, 1H), 6.68 (t, 1H), 6.59 (d, 1H), 3.54 (s, 3H), 3.51 (s, 3H), 2.37 (s, 3H). MS (ES, MH^+) $m/e = 406.09$.

2-(4-Methylphenyl)-3-phenyl-5-(4-methoxyphenylamino)-[1,2,4]thiadiazol-2-ium bromide (3a). To a solution of imidoylthiourea **7a** (2.00 g, 5.33 mmol) in 15 mL of anhydrous chloroform was slowly added bromine (330 μL, 6.40 mmol). After stirring for 16 h, the solvent was evaporated, and the resulting solid was washed with anhydrous ethyl ether. The crude product was recrystallized from a solution of 20% water in ethanol to yield the product **3a** as a yellow solid (1.81 g, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.78 (s, 1H), 7.80 (d, 2H), 7.58 (d, 2H), 7.51 (d, 1H), 7.39 (t, 2H), 7.27 (m, 2H), 7.19 (d, 2H), 6.96 (d, 2H), 3.84 (s, 3H), 2.45 (s, 3H); MS (ES, MH^+) $m/e = 374.25$.

Compounds **3b** and **3c** were prepared in a similar fashion to give yellow solids.

3b (60% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.44 (s, 1H), 7.60 (d, 2H), 7.36 (t, 1H), 7.12–7.32 (m, 6H), 7.04 (m, 2H), 6.88 (t, 1H), 6.80 (d, 1H), 3.60 (s, 3H), 2.34 (s, 3H); MS (ES, MH^+) $m/e = 374.25$.

3c (79% of yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 12.45 (s, 1H), 7.77 (d, 2H), 7.57 (d, 1H), 7.45 (t, 1H), 7.37 (t, 1H), 7.18–7.24 (m, 3H), 7.12 (t, 1H), 6.92 (t, 1H), 6.88 (t, 1H), 6.76 (d, 1H), 3.68 (s, 3H), 3.53 (s, 3H), 2.35 (s, 3H); MS (ES, MH^+) $m/e = 404.12$.

2-(2-Methylphenyl)-3-phenyl-5-(4-methoxyphenylamino)-[1,2,4]thiadiazoline (8). To a solution of imidoylthiourea **7a** (1.00 g, 2.67 mmol) in 10 mL of anhydrous chloroform was added *N*-chlorosuccinimide (356 mg, 2.67 mmol). The reaction mixture was then stirred for 16 hrs, and then diluted to 50 mL of chloroform solution and washed twice with saturated aqueous $NaHCO_3$ solution. The organic layer was dried over $MgSO_4$ and the remaining solvent was removed under vacuum. The crude product was washed by hexane to yield the product **8** as a white solid (457 mg, 46%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.59 (d, 2H), 7.41 (t, 1H), 7.30 (t, 2H), 6.98–7.10 (m, 6H), 6.90 (d, 2H), 3.79 (s, 3H), 2.32 (s, 3H); MS (ES, MH+) $m/e = 374.25$.

2-(2-Methylphenyl)-3-phenyl-5-*N*-methyl-*N*-(4-methoxyphenyl)amino][1,2,4]thiadiazolium triflate (9). To a solution of **8** (100 mg, 0.268 mmol) in 10 mL of anhydrous DCM was added slowly methyl triflate (36.0 uL, 0.322 mmol). After stirring for 1.5 h, the reaction was stopped. After removing the solvent under vacuum, the crude product was washed with hexane and then dried in high vacuum to yield **9** as a yellow solid (133 mg, 92%). ^{1}H NMR (300MHz, $CDCl_3$) δ 7.62 (4H), 7.45 (1H), 7.32 (4H), 7.20 (2H), 7.06 (2H), 3.87 (3H), 3.82 (3H), 2.28 (3H); ^{13}C NMR (300MHz, $CDCl_3$) δ 180.61 (1C), 169.49 (1C), 161.26 (1C), 141.86 (1C), 135.74 (1C), 133.04 (1C), 131.65 (1C), 130.93 (4C), 128.74 (2C), 127.19 (2C), 126.70 (2C), 126.25 (1C), 116.62 (2C), 55.75 (1C), 41.56, (1C), 21.33 (1C); MS (ES, MH+) $m/e = 388.26$. Compound **9** was also characterized by COSY (correlated spectroscopy) and HMBC (hetero multibond coupling) NMR techniques.

Melanocortin MC4 receptor binding assay. The melanocortin receptor MC-4-membrane was purchased from Receptor Biology, Inc. and coupled to wheat germ agglutinin coated polyvinyl toluene-Scintillation Proximity Assay beads (Amersham Pharmacia Inc.) for 30 min at 25 °C. Into each well of a 96-well Opti plate (Packard), 2.5 μg of membrane and 0.25 mg of beads were mixed in a volume of 100 μL media. The media was 50 mM HEPES, pH 7.4 containing 0.1% bovine serum albumin, 2 mM $CaCl_2$, 2 mM $MgCl_2$ and protease inhibitors (Bohreinger Mannheim). Test compounds (1.5 μL) at 1 mM in 30% DMSO—50 mM HEPES, pH 7.4 buffer was added to separate wells on the plate and evaluated at four doses ($n = 2$ or 3). Radioactive ligand ^{125}I-NDP-melanocyte stimulating

hormone (NEN, 2000 Ci/mmol) was added to each well (48.5 μL per well, 40 pM final concentration). The plate was then sealed and incubated for 16 h at 25 °C. NDP-Melanocyte stimulating hormone peptide and α-melanocyte stimulating hormone peptide (Palomar Research Inc, 1 μM) were used as reference inhibitor compounds to define non-specific binding (N). Total binding (T) was defined using 30% DMSO–50 mM HEPES, pH 7.4 buffer. Bound radioactivity for each well (Y) measured at counts per min (cpm) in a TopCount (Packard, CA) was $[(T-Y)/(T-N)]\times 100\%$

Cyclic adenosine monophosphate (cAMP) stimulation assay. Human Bowes melanoma cells expressing human melanocortin MC-4 receptor were grown to confluence in a 24-well culture plate. The cells were washed once with Hanks' solution and each well received 0.5 mL of Hanks' solution containing 1 uM isobutylmethyl xanthine. Test compounds were added to the appropriate test wells. NDP-melanocyte stimulating hormone peptide (1 μM) was added to the positive control wells while negative control wells received vehicle of 30% DMSO–50 mM HEPES, pH 7.4 buffer. The plate was incubated at 37 °C and 5% CO_2 for 30 min. The supernatant was discarded and the cells were washed twice with Hank's solution. Ice cold ethanol (80%, 0.5 mL) was added to each well and the plate was incubated at 4 °C for 30 min to extract cyclic AMP. Cyclic AMP content was measured using the NEN Flashplate kit. A melanocortin receptor agonist is defined as a test compound which resulted in a significant increase in cAMP production compared to vehicle control in this assay.

G-Protein activation assay. For each assay, membranes expressing the melanocortin MC-4 receptor (5 μg) were incubated for 5 min at 25 °C with 0.5 nM ^{35}S-GTPγS in 100 μL of 25 mM HEPES buffer, pH7.5 containing 100 mM NaCl, protease inhibitors, 0.5 μM GDP, 5 mM 2-mercaptoethanol, 1 mM $MgCl_2$ together with an agonist such as the test compound, 1 μM of NDP-melanocyte stimulating hormone or a combination of NDP-melanocyte stimulating hormone and test compound. Basal ^{35}S-GTPγS binding was defined by 10 mM HEPES, pH 7.4 buffer containing 30% DMSO. The reaction was terminated by addition of 50 μL of termination buffer containing 25 mM HEPES, pH7.5, 20 mM $MgCl_2$, protease inhibitors, 100 μM GDP, 100 μM GTP, 5 mM 2-mercaptoethanol with detergents (0.5% digitonin, 0.2% sodium deoxycholate, and 0.5% NP-40). The membranes were solubilized for 30 min at 25 °C. The ^{35}S-GTPγS bound Gαs protein was immunoprecipitated using anti-Gαs (Santa Cruz Biotech Inc., 0.5 μg) that would be linked to anti-rabbit IgG protein A conjugated SPA (Amersham Pharmacia Inc.). Bound radioactivity was measured in a Topcount. Non-specific ^{35}S-GTPγS binding was defined by ^{35}S-GTPγS immunoprecipitated by normal rabbit IgG (Sigma Inc., 0.5 μg).

Rodent feeding: food intake in food-deprived rats (MC-4). Male Long–Evans rats (180–200 g) were housed individually and maintained on a once-a-day feeding schedule for five days following quarantine to allow the animals to acclimate to feeding on powdered chow (#5002 PMI Certified Rodent Meal) during the allotted time. The chow was made available in an open jar, anchored in the cage by a wire, with a metal follower covering the food to minimize spillage, and water was available ad-libitum. Animals were fasted for 18 h prior to testing. At the end of the fasting period, animals were administered either a test compound or vehicle. Vehicle and test compound were administered either orally (5 mL/kg) 60 min prior to the experiment, subcutaneously (1 mL/kg) 30 min prior to the experiment, or intraperitoneally (1 mL/kg) 30 min prior to the experiment. Test compounds were administered orally as a suspension in aqueous 0.5% methylcellulose-0.4% Tween 80, or intraperitoneally as a solution or suspension in PEG 200; compound concentrations typically ranged from 1 to 100 mg/kg, preferably from 10 to 30 mg/kg. Food intake was measured at 2, 4, and 6 h after administration by weighing the special jar containing the food before the experiment and at the specified times. Upon completion of the experiment, all animals were given a 1-week washout period before re-testing.

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