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Identification and characterization of pyrrolidine diastereoisomers as potent functional agonists and antagonists of the human melanocortin-4 receptor

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Abstract—A series of *trans*-4-phenylpyrrolidine-3-carboxamides were synthesized and characterized as potent ligands of the human melanocortin-4 receptor. Interestingly, a pair of diastereoisomers **13b** displayed potent functional agonist and antagonist activity, respectively. Thus, the 3*S*,4*R*-pyrrolidine **13b-1** possessed a K_i of 1.0 nM and an EC_{50} of 3.8 nM, while its 3*R*,4*S*-isomer **13b-2** exhibited a K_i of 4.7 and an IC_{50} of 64 nM. Both compounds were highly selective over other melanocortin receptor subtypes. The MC4R agonist **13b-1** also demonstrated efficacy in a diet-induced obesity model in rats.

The melanocortin receptor family belongs to the G-protein-coupled receptor (GPCR) superfamily and consists of five receptor subtypes that are highly expressed in the brain and other tissues and are regulated by the endogenous peptide agonists, melanocyte-stimulating hormones (α-, β-, and γ-MSH) and adrenocorticotropic hormone (ACTH), and by the endogenous antagonists, agouti-protein and agouti-related protein.[1] The melanocortin-4 receptor (MC4R) plays an important role in the regulation of feeding behavior,[2] and thus, a potent and selective MC4R agonist with brain penetration could perhaps be useful in the treatment of obesity.[3]

Several small molecule MC4R agonists from different chemical classes have been discovered.[4] For example, pyrrolidine derivatives exemplified by **1** (Fig. 1) have been reported by Ujjainwalla to be potent and selective at MC4R.[5] We have previously reported that, by combining the *trans*-4-arylpyrrolidine-3-carbonyl moiety in **1** with the piperazinebenzylamine portion in **2**, presented in a series of 3-aryl-2-methylpropionamide MC4R antagonists,[6] potent MC4R agonists such as **3** have been identified.[7] Here we report the optimization of this series of compounds and the in vitro characterization of the diastereoisomers **13b-1** and **13b-2** as a potent MC4R agonist and antagonist, respectively. In vivo studies of the agonist **13b-1** in feeding and obesity animal models are also discussed.

The *trans*-*N*-isopropyl-4-arylpyrrolidin-3-ylcarboxylic esters **6** were synthesized from the corresponding methyl cinnamates and *N*-(trimethylsilylmethyl)-*N*-(methoxymethyl)isopropylamine **5**, which was prepared by the reaction of isopropylamine with trimethylsilylmethyl chloride **4**, followed by formaldehyde and methanol, using a procedure similar to that described in the literature.[8] Hydrolysis of **6** under basic conditions gave the

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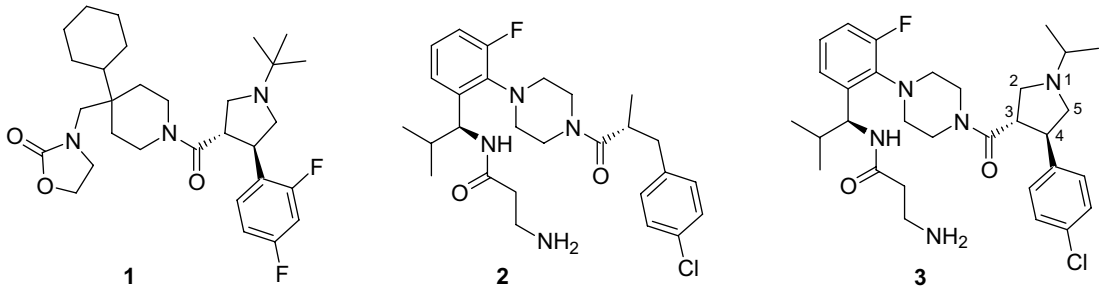


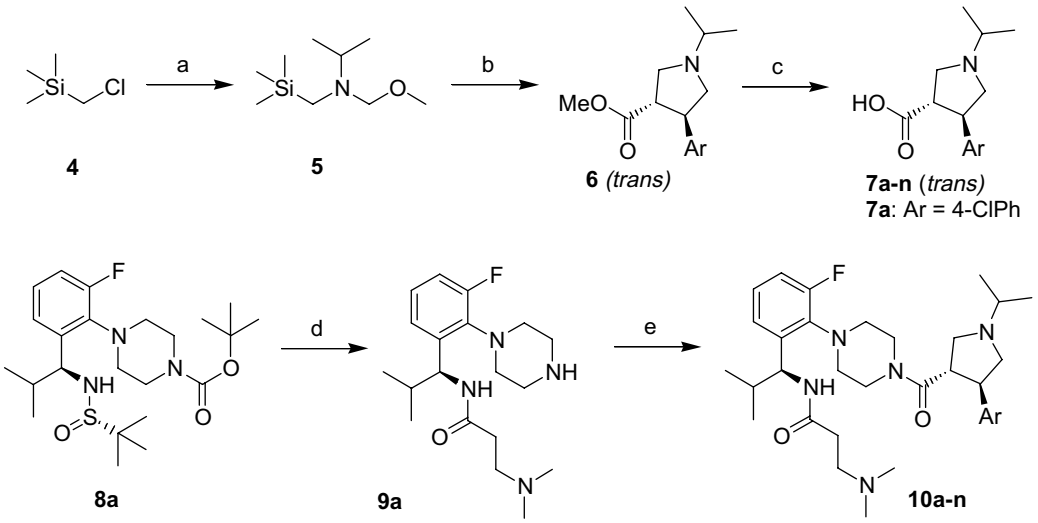
Figure 1. Chemical structures of compounds **1**–**3**.

acids **7a**–**n** in good yields. The *cis*-isomer *cis*-**7a** was synthesized using the same procedure but from methyl *cis*-4-chlorocinnamate.[9] The β-alanine derivative **9a** was prepared from the benzylamine **8a**.[10] Thus, de-sulfination of **8a** with HCl in methanol afforded an amine intermediate which was coupled with *N*,*N*-dimethyl-β-alanine under standard peptide coupling conditions to give the amine **9a** after Boc-deprotection with trifluoroacetic acid. Coupling reactions of the *trans*-pyrrolidinecarboxylic acids **7a**–**n** with the amine **9a** provided the amides **10a**–**n** in various yields (Scheme 1).

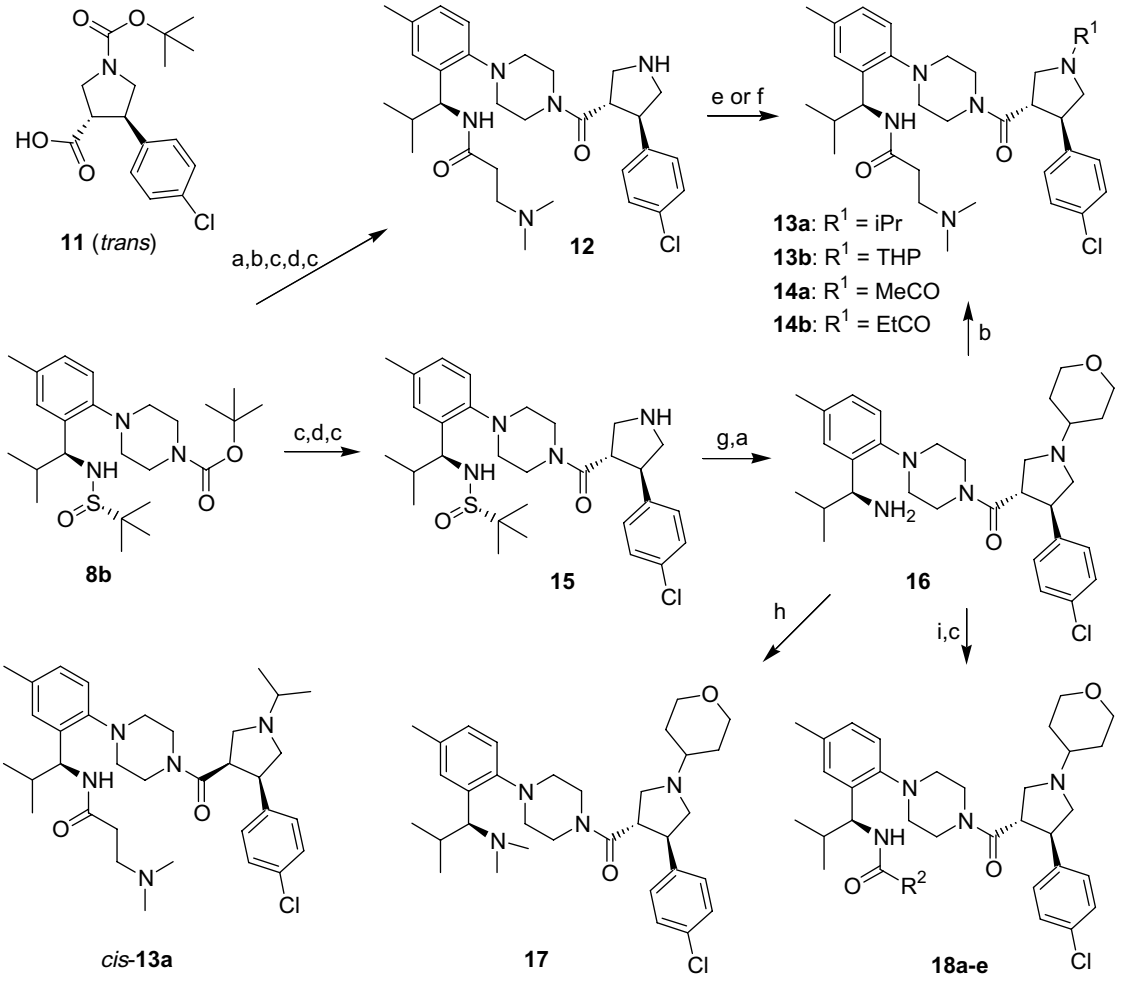
Compound **13a** was synthesized from the piperazinebenzylamine **8b** as shown in Scheme 2. Treatment of **8b** with HCl in methanol afforded the corresponding amine which was coupled to *N*,*N*-dimethylaminopropionic acid to provide the amide intermediate **9b** after TFA-treatment. A coupling reaction of **9b** with *trans*-*N*-Boc-4-(4-chlorophenyl)pyrrolidine-3-carboxylic acid **11** gave the intermediate **12** after Boc-deprotection. Reductive alkylation of **12** with acetone afforded the isopropylamine **13a**. *cis*-**13** was synthesized by directly coupling **9b** with *cis*-**7a** using a procedure similar to that for **10a**. The amides **14a**–**b** were obtained by the reaction of **12** with acetyl or propionyl chloride in the presence of triethylamine.

Alternatively, a coupling reaction of **11** with the amine generated from **8b** with TFA afforded the secondary amine **15** after Boc-deprotection. Reductive alkylation of **15** with tetrahydropyran-4-one provided the tetrahydropyran-4-yl (4-THP) amine **16** after HCl/MeOH treatment. The single isomers **16-1** and **16-2** were obtained, respectively, using the same protocol from the single diastereoisomers **11-1** (3*S*,4*R*) and **11-2** (3*R*,4*S*) that were obtained from the separation of *trans*-*N*-benzylpyrrolidinecarboxylic acid using (4*R*)- or (4*S*)-benzyloxazoline as previously described.[11] Dimethylation of **16** using formaldehyde under reductive conditions gave the tertiary benzylamine **17** in 51% yield. A coupling reaction of **16** with *N*,*N*-dimethylaminopropionic acid provided the amides **13b**. Two single isomers **13b-1** and **13b-2** were obtained from **16-1** and **16-2**, respectively, using the same method. Finally, coupling reactions of **16** with various *N*-Boc amino acids afforded the amides **18** after Boc-deprotection.

The final compounds were tested in a binding assay in HEK293 cells expressing human melanocortin-4 receptors using [^{125}I]-NDP-MSH as the radiolabeled ligand. Compounds with high affinity were also tested in a cAMP functional assay as previously described.[12] The results are summarized in Tables 1–3.



Scheme 1. Reagents and conditions: (a) i—*i*-PrNH$_2$/60 °C, 16 h, 73%, 37% CH$_2$O/0 °C to rt, 0.5 h, then MeOH, 62%; (b) *trans*-ArCH=CHCOOMe/CH$_2$Cl$_2$/0 °C to rt, 4 h, various yields; (c) NaOH/MeOH/H$_2$O/rt, 8 h, >90%; (d) i—HCl/MeOH/rt, 1 h; ii—Me$_2$NCH$_2$CH$_2$COOH/EDC/HOBt/Et$_3$N/CH$_2$Cl$_2$/rt, 8 h, TFA/CH$_2$Cl$_2$/rt, 1 h, 65%; (e) **7**/EDC/HOBt/Et$_3$/N/CH$_2$Cl$_2$/rt, 16 h, 5–80%.



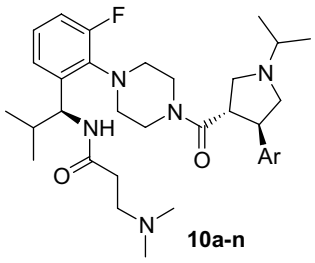
Scheme 2. Reagents and conditions: (a) HCl/MeOH/rt, 1 h; (b) $Me_2NCH_2CH_2COOH$/EDC/HOBt/Et_3N/DMF/rt, 16 h, ~60%; (c) TFA/CH_2Cl_2/rt, 1 h, >90%; (d) **11**/EDC/HOBt/Et_3N/DMF/CH_2Cl_2/rt, 16 h, 60–88%; (e) acetone/$NaBH(OAc)_3$/CH_2Cl_2/rt, 1 h, 64%; (f) MeCOCl or EtCOCl/Et_3N/CH_2Cl_2/rt, 1 h, 90%; (g) tetrahydropyran-4-one/$NaBH(OAc)_3$/AcOH/CH_2Cl_2/rt, 8 h, 36%; (h) CH_2O/$NaBH(OAc)_3$/CH_2Cl_2/rt, 2 h, 51%; (i) R^2COOH/EDC/HOBt/Et_3N/CH_2Cl_2/rt, 1–8 h, 20–60%.

For the 4-arylpyrrolidines **10a**–**n**, the 4-chlorophenyl compound **10a** possessed good binding affinity (K_i = 5.3 nM) and high agonist potency (EC_{50} = 20 nM) with full intrinsic activity (IA = 103%) compared to the internal standard α-MSH. The 3-chlorophenyl analog **10b** (K_i = 370 nM) was 70-fold less potent in binding affinity compared to **10a**, and the 3-methoxyphenyl **10c** was 10-fold less potent than **10b**. The 2,4-dichlorophenyl **10f** had a comparable K_i value to **10a**, while the 2-fluoro-4-trifluoromethylphenyl **10g** was about 10-fold less potent. However, **10f** displayed very low efficacy (IA = 24%), even at a 10 μM concentration. In a functional antagonist assay, **10f** dose-dependently inhibited α-MSH-stimulated cAMP production with an IC_{50} of 560 nM. The regio-isomer of **10f** (**10k**, K_i = 51 nM, EC_{50} = 340 nM, IA = 47%) had moderate binding affinity but was a partial agonist, while the 3-chloro-4-trifluoromethylphenyl analog **10j** (K_i = 260 nM, EC_{50} = 410 nM, IA = 78%) was a moderately potent agonist. All other analogs **10** showed poor binding affinity, including the 3-furyl compound **10n** (Table 1).

The 4-methylphenylpiperazine **13a** (K_i = 4.7 nM, EC_{50} = 16 nM, IA = 102%) displayed binding affinity and agonist potency very similar to the 6-fluoro analog **10a**. Its des-isopropyl analog (**12**, K_i = 7.2 nM, EC_{50} = 79 nM, IA = 72%) possessed a slightly reduced binding affinity, agonist potency, and efficacy compared to **13a**, indicative of a role of this small but lipophilic *N*-isopropyl group. *cis*-**13a** (K_i = 90 nM) possessed much lower binding affinity than its *trans*-isomer **13a**, suggesting a stereo-preference of the 4-chlorophenyl group. The *N*-tetrahydropyran **13b** (K_i = 1.4 nM, EC_{50} = 10 nM, IA = 121%) exhibited high binding affinity as well as functional activity. The *N*-acyl pyrrolidines **14a** and **14b** also showed high binding affinity and good agonist potency, indicative of a minimal role of the basic nitrogen in **13** (Table 2).

We have previously shown that the diastereoisomers **16-1** and **16-2** were a functional agonist and antagonist, respectively (Table 3).[11] Similar to this pair, the individual stereoisomers **13b-1** and **13b-2** displayed similar binding affinities but different functional activities. Thus, **13b-1** (K_i = 1.0 nM) was a potent functional agonist with an EC_{50} of 3.8 nM and intrinsic activity of 122%. In contrast, **13b-2** (K_i = 4.7 nM) was only able to stimulate cAMP accumulation to about 10% of the

Table 1. Binding affinity of various *trans*-4-arylpyrrolidine-3-carboxamides **10a–n**[a]

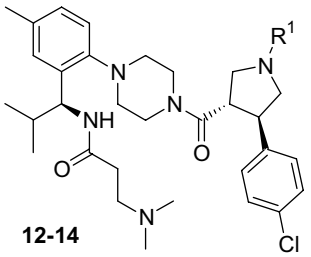


10a-n

Compound	Ar	K_i (nM)	EC_{50}[b] (nM)
10a	4-ClPhC_6H_4	5.3	20 (103%)
10b	3-ClC_6H_4	370	
10c	3-MeOC_6H_4	3700	
10d	3-MeC_6H_4	470	
10e	3-FPhC_6H_4	560	
10f	2-F,4-$CF_3C_6H_3$	61	
10g	2,4-$Cl_2C_6H_3$	7.5	(24%)[c]
10h	2,5-$F_2C_6H_3$	2050	
10i	3,4-$F_2C_6H_3$	290	
10j	3-Cl,4-FC_6H_3	260	410 (78%)
10k	3-F,4-$CF_3C_6H_3$	51	340 (47%)
10l	3,5-$F_2C_6H_3$	7800	
10m	3,4,5-$F_3C_6H_2$	250	
10n	3-Furyl	850	

[a] Data are average of two or more independent measurements.
[b] Intrinsic activity relative to α-MSH reported in parentheses.
[c] Dose-dependent inhibition of α-MSH-stimulated cAMP release with IC_{50} = 560 nM.

Table 2. SAR of the *N*-group of pyrrolidine derivatives **13–14** at *h*MC4R[a]



12-14

Compound	R^1	K_i (nM)	EC_{50}[b] (nM)
12	H	7.2	79 (72%)
13a	*i*-Pr	4.7	16 (102%)
cis-**13a**	*i*-Pr	90	[c]
13b	4-THP	1.4	10 (121%)
14a	MeCO	7.6	44 (100%)
14b	EtCO	4.6	27 (129%)

[a] Data are average of two or more independent measurements.
[b] Intrinsic activity relative to α-MSH in cAMP stimulation.
[c] Not determined.

α-MSH level at 10 μM concentration. In the functional antagonist assay, **13b-2** dose-dependently inhibited α-MSH-stimulated cAMP accumulation with an IC_{50} of 64 nM (Fig. 2).[13]

While the binding affinity of the agonist **13b-1** was about 5-fold better than that of the antagonist **13b-2**, **16-1** and **16-2** exhibited similar affinity. Unlike **13b** (IA = 121%), the mixture of the *trans*-isomers **16** only displayed partial intrinsic activity (IA = 36%). Its *N*,*N*-dimethyl analog **17** (K_i = 5.3 nM, IA = 35%) also showed partial agonism. Other derivatives of **16** with an additional amino acid side-chain possessed high binding affinity and high intrinsic activity (compounds **18a–c**), but the two alanine derivatives **18d** and **18e** were partial agonists with high potency (Table 3). The factor contributing to full or partial receptor activation for these *trans*-isomers **18a–e** is unclear. The single isomer agonist **13b-1** was 11-fold more potent than the agonist **16-1**, suggesting that the aminopropionyl side-chain strengthens the interaction of the agonist **13b-1** with the receptor.

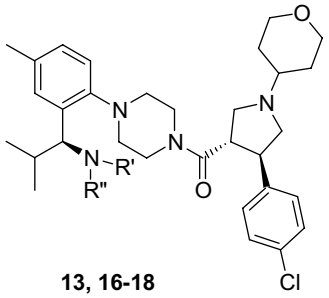
In competition binding assays, both **13b-1** and **13b-2** were highly selective for *h*MC4R over other melanocortin receptor subtypes (Table 4). These two compounds were also characterized for their pharmacokinetic properties in rats. After an intravenous injection of 2.5 mg/kg, **13b-1** displayed a high plasma clearance of 129 mL/min kg and a very high volume of distribution (V_d = 80 L/kg), resulting in a long half-life ($t_{1/2}$ = 7.1 h) in this species (Table 4).

After an oral gavage at a 10 mg/kg dose, the maximal plasma concentration (C_{max}) of 14 ng/mL appeared at 2.7 h, and the AUC value was 114 ng/mL h. The oral bioavailability was 10%. Despite its high tissue distribution, which might be related to its dibasic structure, **13b-1** had delayed brain penetration. The whole brain concentration was below the limit of detection at the 1-h time point post po dosing and was only 19 ng/g at the 4-h time point. This resulted in a brain/plasma ratio of 3.0 due to a very low plasma concentration. The PK parameters of **13b-2** were very similar to those of its stereoisomer **13b-1** (Table 5).

In an in vitro Caco-2 assay, **13b-1** had a P_{app} of 1.6 nm/s from the apical (a) to basolateral (b) direction and a (b to a)/(a to b) ratio of 23, demonstrating high efflux which might be associated with P-glycoprotein transporter activity. Compound **13b-1** had a measured log *D* value of 2.6 using a shake-flask method. Its high molecular weight of 638 and an efflux mechanism are probable causes for its limited brain penetration.

The potent and selective agonist **13b-1** was also evaluated in vivo. The effect of this compound on food intake over 24 h was first tested in DIO rats that were food deprived overnight and on the test day. One hour after administration of **13b-1** at 3, 10 or 30 mg/kg via intraperitoneal (ip) injection with vehicle (sterile water), food was returned and intake measured at defined intervals over a 24-h period. Food intake was significantly suppressed compared to vehicle-treated rats at all time points and all doses (Fig. 3, $p < 0.05$). In this study, fenfluramine was used as a positive control and it significantly suppressed feeding at the measured time points as expected.

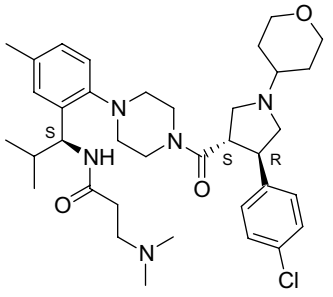
Table 3. SAR of the *N*-group at the benzylamine derivatives **13** and **17**–**18**[a]



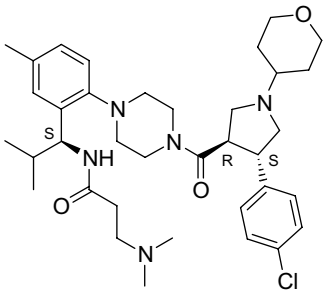
13, 16-18

Compound	R′	R″	K_i (nM)	EC_{50}[b] (nM)
16	H	H	12	(36%)
16-1 (3*S*,4*R*)			11	24 (104%)
16-2 (3*R*,4*S*)			8.6	(18%)[c]
17	Me	Me	5.3	(35%)
13b	$COCH_2CH_2NMe_2$	H	1.4	10 (121%)
13b-1 (3*S*,4*R*)			1.0	3.8 (122%)
13b-2 (3*R*,4*S*)			4.7	(10%)[d]
18a	$COCH_2CH_2NH_2$	H	2.2	6.0 (107%)
18b	$COCH_2NH_2$	H	2.0	9.1 (114%)
18c	$COCH_2NHMe$	H	2.1	19 (84%)
18d	*R*-COCH(Me)NH_2	H	7.3	17 (56%)
18e	*S*-COCH(Me)NH_2	H	1.0	5.2 (38%)

[a] Data are average of two or more independent measurements.
[b] Intrinsic activity relative to α-MSH is indicated in parentheses.
[c] Dose-dependent inhibition of α-MSH-stimulated cAMP release with IC_{50} of 65 nM.
[d] Dose-dependent inhibition of α-MSH-stimulated cAMP release with an IC_{50} of 64 nM.



13b-1 (*S,S,R*-isomer):
K_i = 1.0 nM, EC_{50} = 3.8 nM, IA = 122%



13b-2 (*S,R,S*-isomer):
K_i = 4.7 nM, IA = 10%, IC_{50} = 64 nM

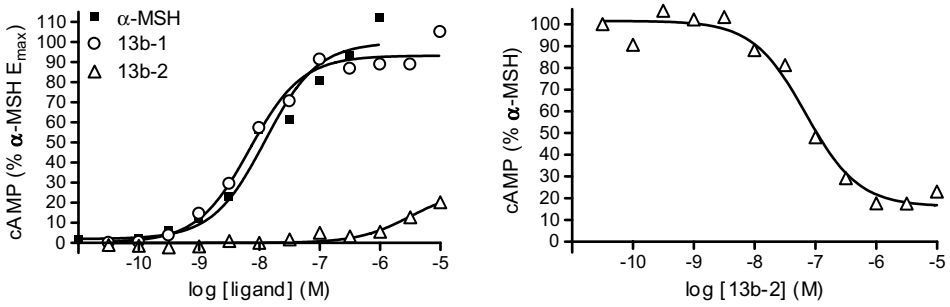


Figure 2. Chemical structures of **13b-1** and **13b-2** and their functional activity in cAMP assays.

Based on the robust effects observed with ip administration, the effects of oral (po) administration of **13b-1** were also examined in the same model. Food intake following oral administration of **13b-1** was suppressed by only

Table 4. Binding affinity (K_i, nM) of **13b-1** and **13b-2** at the melanocortin receptors[a]

Compound	MC1R	MC3R	MC4R	MC5R
13b-1	(38%)[b]	260	1.0	800
13b-2	120	1300	4.7	530

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

Table 5. Pharmacokinetic parameters of **13b-1** and **13b-2** in rats[a]

Compound	**13b-1**	**13b-2**
iv dose (mg/kg)	2.5	2.5
CL (mL/min kg)	129	82
V_d (L/kg)	80	75
$t_{1/2}$ (h)	7.1	11
AUC (ng/mL h)	307	455
po dose (mg/kg)	10	10
C_{max} (ng/mL)	14	17
T_{max} (h)	2.7	2.0
AUC (ng/mL h)	114	161
F (%)	10	14
C_{brain} (ng/g) at 1, 4 h	0, 19	0, 18
C_{brain}/C_{plasma}	0, 3.0	0, 1.0

[a] Data are average of three animals.

30 mg/kg, the highest dose tested (and fenfluramine) at all the time points tested ($p < 0.05$).

Given the positive effects on acute feeding behavior after a single oral dose of **13b-1**, we next evaluated how chronic administration of this selective MC4 agonist would alter body weight and other related parameters in the DIO rat.[14] Figure 4 depicts the effects of daily **13b-1** treatment on food intake and body weight. Both fenfluramine and 30 mg/kg of **13b-1** significantly suppressed food intake on all test days. The 10 mg/kg dose of **13b-1** inhibited feeding on day 1, 2, and 6 while the 3 mg/kg dose inhibited feeding on day 6. Compared to vehicle-treated controls, body weight was also significantly reduced with fenfluramine treatment on all days and with 30 mg/kg of **13b-1** on days 2–7.

After 7 days of **13b-1** administration, body composition (Rat EchoMRI, EchoMRI, TX) and fasting glucose (OneTouch Ultra, LifeScan, CA) were measured. Relative to baseline measurements, compound **13b-1** produced a dose-dependent increase in fat loss (Fig. 5) but did not significantly affect lean mass. These body composition results demonstrate that body weight loss due to **13b-1** treatment occurred solely as a result of fat loss, while lean mass was spared. Furthermore,

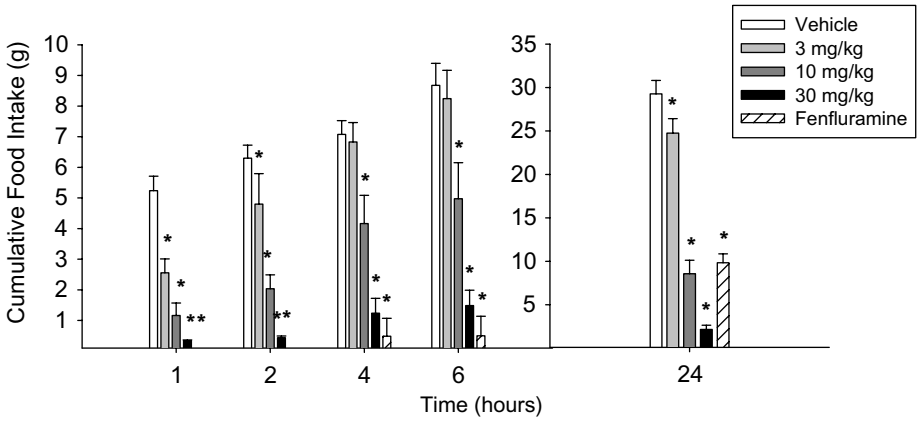


Figure 3. Effect of **13b-1** (ip) on cumulative food intake in 24-h food deprived male DIO rats.

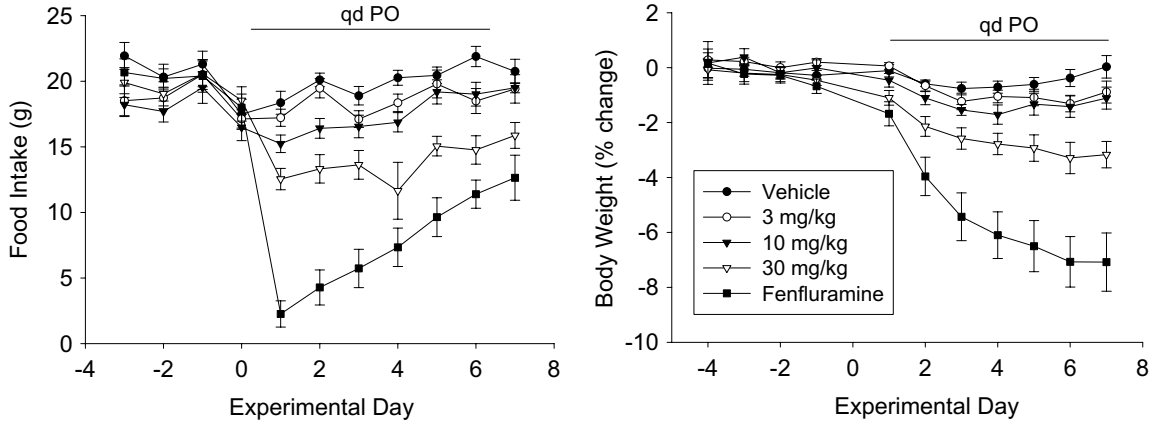


Figure 4. Effect of **13b-1** (po) administered once a day for 7 days on food intake and body weight change in male DIO rats.

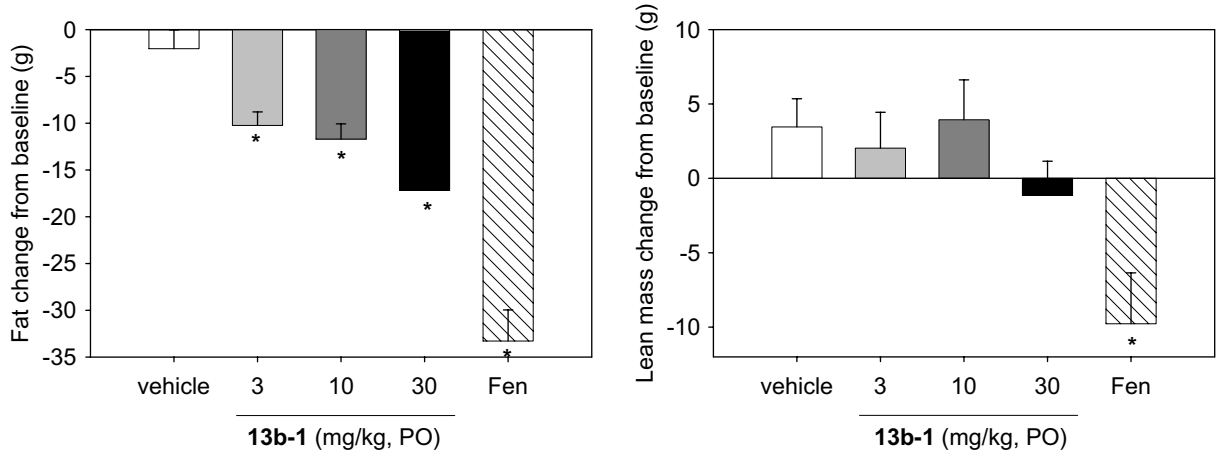


Figure 5. Effect of 7 days of oral administration of **13b-1** on fat and lean mass change in male DIO rats ($^{*}p < 0.05$).

13b-1 administration seemed to positively affect glucose regulation. Fasting glucose was slightly but significantly decreased in the DIO rats given the 30 mg/kg dose of **13b-1** (Fig. 6). It is unlikely that this effect is simply a correlate of weight loss considering the fenfluramine-treated animals lost more weight but did not show decreased blood glucose levels.

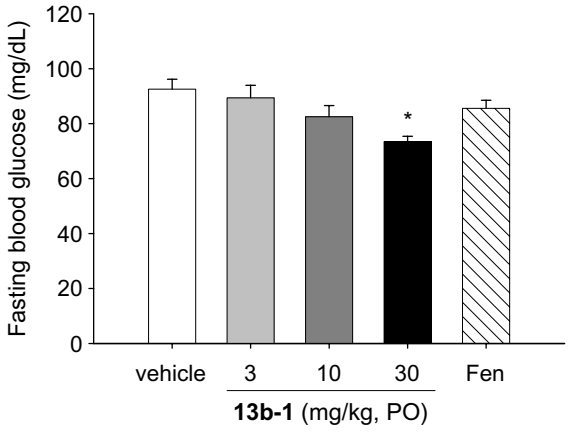


Figure 6. Effect of 7 days of oral administration of **13b-1** (po) on fasting blood glucose in male DIO rats ($^{*}p < 0.05$).

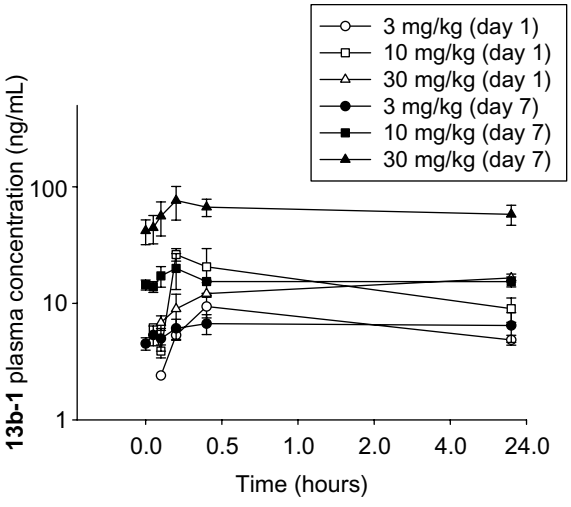


Figure 7. Time–concentration curves for compound **13b-1** after oral administration on day 1 and day 7 in the chronic feeding study in DIO rats.

In the chronic study, the plasma concentrations of the compound were also monitored in a satellite group of DIO rats, and the results are shown in Figure 7. On day 1, plasma concentrations were relatively proportional to the dose (3, 10, and 30 mg/kg). On day 7, the plasma exposure was increased by about 5-fold compared to day 1, suggesting accumulation of this compound. The brain/plasma ratio also increased from about 4 to over 10. This phenomenon was probably associated with its high volume of distribution in rats.

In conclusion, we have studied a series of *trans*-4-arylpyrrolidine-3-carboxamides as potent MC4R agonists. Several potent compounds were identified. It was observed that the 3*S*,4*R*-isomer of **13b-1** was able to activate the MC4 receptor with high potency. In contrast, its 3*R*,4*S*-isomer **13b-2** possessed poor intrinsic activity in the cAMP-stimulation assay and functioned as an antagonist in the cAMP-inhibition assay. The potent and selective agonist **13b-1** was characterized in vivo and demonstrated efficacy in a dose-dependent manner in a chronic DIO study.

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13. Functional efficacy of compound **13b-1** (agonist) and compound **13b-2** (antagonist): accumulation of cAMP production in CHO cells transfected with *h*MC4R was measured and analyzed using an ELISA. Data are from representative experiments performed 2–8 times with similar results. (left) Compound **13b-1**, compound **13b-2**, and α-MSH stimulation of cAMP production, where 100% cAMP is defined as the E_{max} of α-MSH-stimulated cAMP production. (right) Compound **13b-2** antagonism of α-MSH-stimulated cAMP production, where 100% cAMP is defined as stimulation of cAMP production by 10 nM α-MSH ($\sim EC_{50}$) in the absence of antagonist.
14. Separate groups of DIO rats were orally dosed once a day (1 h prior to lights out) with either vehicle, fenfluramine, 3, 10, or 30 mg/kg of **13b-1**. Food intake (corrected for spillage) and body weight were measured daily.