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Pyrrolidinones as orally bioavailable antagonists of the human melanocortin-4 receptor with anti-cachectic activity

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Abstract—A series of pyrrolidinones derived from phenylalanines were synthesized as potent antagonists of the human melanocortin-4 receptor. These compounds showed high potencies and selectivities, and several of them had good oral bioavailabilities. In addition, **12e** demonstrated in vivo efficacy in a murine cachexia model.

The melanocortin-4 receptor (MC4R) is a member of the G-protein-coupled receptor (GPCR) superfamily and it plays an important role in regulating feeding behavior and energy homeostasis.[1] Recent studies have shown that MC4R antagonists can increase food intake, and more importantly, reverse lean body mass loss in animal models of cachexia.[2,3] Therefore, a potent and selective MC4R antagonist with good oral bioavailability might be useful in clinical treatment of cachexia.[4]

Potent and selective antagonists of the melanocortin-4 receptor from several chemical classes have been discovered.[5] For example, the amidine **1** (Fig. 1) has been shown by Vos and coworkers, as a MC4R antagonist with CNS penetration, to protect rodent from weight loss induced by tumor.[6] The dipiperazine **2** is also a potent MC4 antagonist, which exhibits efficacy in a anxiety model.[7] We have previously identified a series of α-benzylpropionylpiperazines such as **3a** ($K_i = 25$ nM) as MC4R antagonists with moderate oral bioavailability and high brain penetration in rodents.[8] To further improve the potency and selectivity of these compounds, we have conducted a study by using a nitrogen-containing moiety to replace the α-methyl group of **3a**. These modifications increase potency from the initial phenyl propionyl compounds.[9] Here we report the discovery of a series of potent and selective MC4R antagonists with good oral bioavailability in mice.

Compounds *S*-**7**, **9**–**10** were synthesized from the key intermediates *S*-**5** according to the sequence described in Scheme 1. Thus, coupling reactions of *S*-**4**[10] with *N*-Boc-D(2,4-Cl)phenylalanine, followed by selective deprotection with TFA, gave the primary amines *S*-**5** in good yields. These compounds were then acetylated with acetic anhydride, or coupled with *N*-Boc-β-alanine and *N*-Boc-glycine, to afford intermediates that were fully deprotected using hydrochloric acid to provide compounds *S*-**7**, **9**–**10**. Alternatively, coupling reactions of *S*-**4** with preformed *N*-Boc-β-Ala-D(2,4-Cl)Phe-OH or *N*-Boc-Gly-D(2,4-Cl)Phe-OH, followed by deprotection, afforded *S*-**9**–**10**. A similar procedure was used to prepare *R*-**7**–**10** from the corresponding *R*-**4**. Deprotection of *S*-**5d** (X = 4-CF_3) with HCl gave the diamine **6**. *S*-**5d** was also converted to the piperazinone **11** by the following reaction sequence: reductive alkylation with *N*-Boc-glycinaldehyde; treatment with chloroacetyl

Keywords: Pyrrolidinone; Synthesis; Melanocortin-4 receptor; Antagonist; Pharmacokinetics; Cachexia; Animal model.
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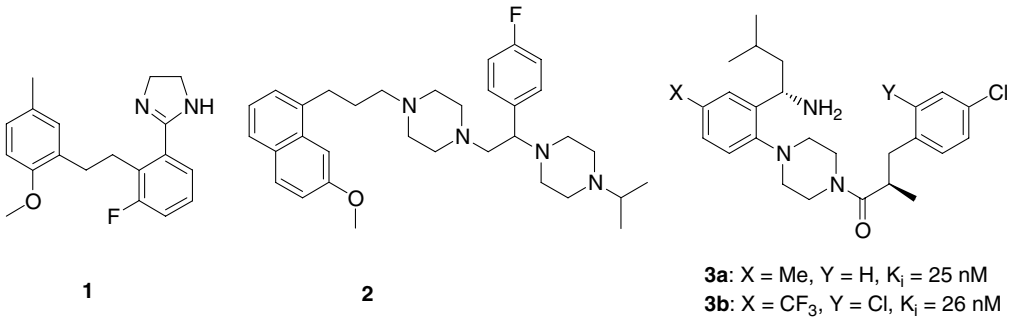
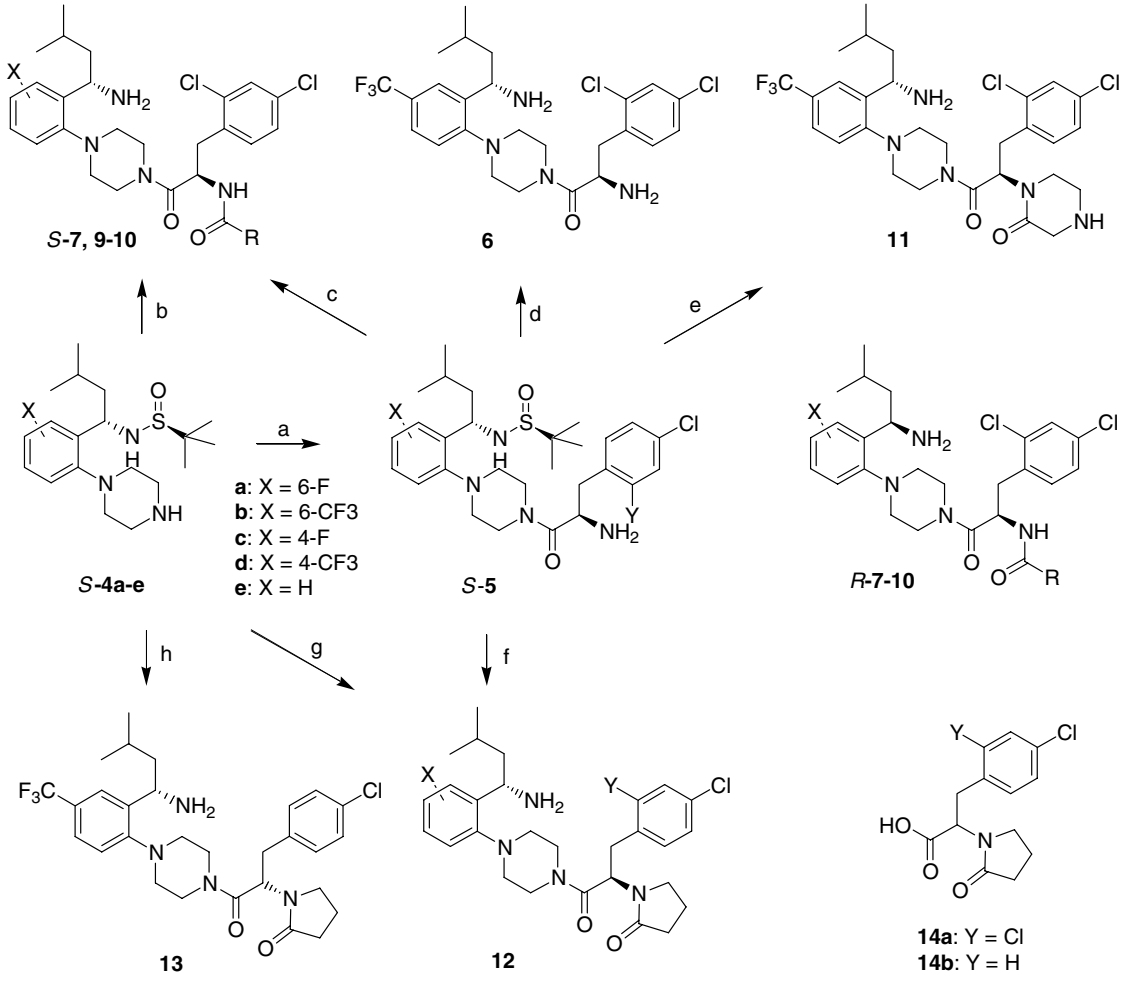


Figure 1. Examples of MC4R antagonists.



Scheme 1. Reagents and conditions: (a) *N*-Boc-D(2-Y,4-Cl)Phe-OH, HBTU, DIEA, DMF, rt, then TFA, CH_2Cl_2, 50–75% over two steps; (b) *N*-Ala-D(2,4-Cl)Phe-OH, HBTU, DIEA, DMF, rt, then HCl, MeOH, about 65% over two steps; (c) RCOOH, EDC, HOBt, rt or Ac_2O, CH_2Cl_2, DIEA, DMAP cat., then HCl, MeOH; (d) HCl, MeOH; (e) i—Boc-$NHCH_2CHO$, $NaBH(OAc)_3$, CH_2Cl_2, rt, 59%; ii—$ClCH_2COCl$, EtOAc, aq $NaHCO_3$, rt; iii—HCl, MeOH, rt, 57% over three steps; (f) i—$HOOCCH_2CH_2CHO$, $NaBH(OAc)_3$, H_2O, THF, rt; ii—THF, Ac_2O, rt; iii—HCl, MeOH, 20–50%; (g) *R*-**14**, HBTU, DIEA, DMF, rt, then HCl, MeOH, rt, about 60% over two steps; (h) *S*-**14b**, HBTU, DIEA, DMF, rt, then HCl, MeOH, rt, 60% over two steps.

chloride under basic conditions; full deprotection under acidic conditions (HCl in MeOH), followed by treatment with aqueous Na_2CO_3 to promote a cyclization.

Pyrrolidinones **12a**–**d** were obtained from reductive alkylations of the amines *S*-**5d** with succinic semialdehyde in the presence of sodium acetoxyborohydride, followed by Ac_2O-promoted lactamization and *tert*-butanesulfinamide deprotection. Compounds **12** could be prepared by reactions of *S*-**4** with pre-assembled pyrrolidinones *R*-**14**[11] under standard peptide coupling conditions. The *S*-configured 4-chlorophenylalanine derivative **13** was prepared by using the D-phenylalanine *S*-**14b**.[12] These compounds were then tested for their binding affinity at

the human MC4 receptor using [^{125}I]-NDP-MSH {[Nle^4,D-Phe^7]α-melanocyte-stimulating hormone} radiolabled ligand as previously reported.[13]

In comparison with 3-(2,4-dichlorophenyl)propionylpiperazine **3b** (K_i = 26 nM), the 2,4-chlorophenylalanine compound **6** (K_i = 39 nM) exhibited similar binding affinity (Table 1). Acetylation of **5** increased its binding affinity by over 20-fold (*S*-**7a**, K_i = 1.8 nM). Incorporation of an amine group (*S*-**7b**–**d**) improved potency slightly from *S*-**7a**. This was different from some other published series, in which a β-alanine seems to be important in this area.[14] These results suggest that an additional amine moiety in the current set of compounds plays a minimal role, probably due to a strong interaction with the receptor from the optimized 'western-side' of the molecule. In addition, *R*-**7c** (K_i = 10 nM) was about 10 times less active than its *S*-isomer, demonstrating a stereo preference at this site of the molecules.

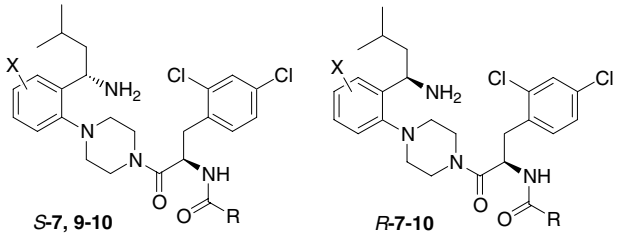
Moving the trifluoromethyl group of *S*-**7** from the 4-position of the phenyl ring to the 6-position (*S*-**9**) resulted in only slight loss in potency, and the ratio between the *S*- and *R*-isomers of **9** was similar to that of **7**. For example, *R*-**9c** (K_i = 29 nM) was about 12-fold less potent than *S*-**9c** (K_i = 2.5 nM).

The 6-fluorophenyl compounds **10** showed some interesting structure-activity relationships. The *S*-acetamide *S*-**10a** had a K_i of 7.5 nM, which was significantly less potent than the β-alanine *S*-**10c**. Interestingly, *R*-**10a** exhibited a similar K_i value to *S*-**10a**. In comparison, *S*-**10c** had a K_i of 0.8 nM, which matched that of *S*-**7c**, but *R*-**10c** was 3-fold less potent than its *S*-isomer *S*-**10c**. These results might suggest that the 6-fluoro affects the dihedral angle between the piperazine plane and the phenyl ring, which has an orthogonal relationship for a closely related analog,[7] and this relationship could be important for the pharmacophore of an active compound.

While compounds such as *S*-**7b**, *S*-**7c**, *S*-**9c**, *S*-**10c**, and *R*-**10c** displayed high binding affinity, they generally possessed high polar surface due to the polar amine and the secondary amide, which is undesirable for a CNS agent.[15] We chose to retain the amide functionality by using a cyclized form. Thus, the piperazinone **11**, derived from the glycine *S*-**7b** via an ethylene bridge, exhibited a K_i of 4.5 nM, which was only about 2-fold less potent than its parent. Similarly, the pyrrolidinone **12a** (K_i = 4.5 nM) also possessed good binding affinity.

The substitution at the western-side phenyl ring of the pyrrolidones (**12a**–**d**) had a minimal effect on binding affinity except for the 4-fluoro group which decreased potency (**12b**, K_i = 26 nM, Table 2). The 4-chlorophenylalanine **12e** on the 4-trifluoromethyl template displayed only 2-fold reduction in potency from the 2,4-dichloro analog **12a**, however, the monochloro compound **12f** was 6-fold less potent than the dichloro analog **12c**, suggesting that the lipophilic 4-trifluoromethyl group of **12e** plays a role in maintaining its potency. Interestingly, the L-(4-chlorophenyl)alanine derivative **13** (K_i = 8.8 nM) possessed similar binding affinity to its D-analog **12e** (K_i = 11 nM). This lack of stereo-preference might indicate that the pyrrolidinone moiety does not have a direct interaction with the receptor. Instead, it helps to orient the 4-chlorophenyl group to be located in a preferred position for its receptor interactions.[16,17] A 'Y' shape conformation for the Tic-D(4-Cl)Phe piperazine of the THIQ MC4R agonist has been demonstrated by its solid-state structure.[18]

Table 1. SAR of amides at the human MC4R

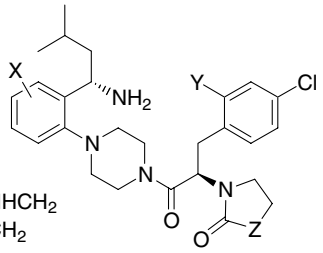


S-**7, 9-10** *R*-**7-10**

Compound	X	R	K_i[a] (nM)
6	4-CF_3		39
S-**7a**	4-CF_3	Me	1.8
S-**7b**	4-CF_3	CH_2NH_2	1.8
S-**7c**	4-CF_3	$CH_2CH_2NH_2$	1.0
R-**7c**	4-CF_3	$CH_2CH_2NH_2$	10
S-**7d**	4-CF_3	4-Piperidinyl	0.8
R-**8a**	4-F	Me	26
R-**9a**	6-CF_3	Me	47
S-**9b**	6-CF_3	CH_2NH_2	3.3
R-**9b**	6-CF_3	CH_2NH_2	49
S-**9c**	6-CF_3	$CH_2CH_2NH_2$	2.5
R-**9c**	6-CF_3	$CH_2CH_2NH_2$	29
S-**10a**	6-F	Me	7.5
R-**10a**	6-F	Me	6.0
S-**10b**	6-F	CH_2NH_2	3.3
R-**10b**	6-F	CH_2NH_2	7.1
S-**10c**	6-F	$CH_2CH_2NH_2$	0.8
R-**10c**	6-F	$CH_2CH_2NH_2$	2.3

[a] Data are average of two or more independent measurements.

Table 2. SAR of heterocycles at the human MC4R



11: Z = $NHCH_2$
12: Z = CH_2

Compound	X	Y	K_i[a] (nM)
11	4-CF_3	Cl	4.5
12a	4-CF_3	Cl	4.5
12b	4-F	Cl	26
12c	H	Cl	6,4
12d	6-F	Cl	9.7
12e	4-CF_3	H	11
13	4-CF_3	H	8.8
12f	H	H	38

[a] Data are average of two or more independent measurements.

Several compounds were further tested for their binding affinities at the other melanocortin receptor subtypes and found to be highly selective. For example, *S*-**10c** had K_i values of 450, 0.8, and 240 nM at the MC3R, MC4R, and MC5R, respectively (Table 3). Compound **12e** had the best selectivity (~100-fold) among all analogs tested, and it did not have appreciable binding affinity at the MC1 receptor. None of the compounds listed in Tables 1 and 2 exhibited significant stimulation of cAMP release in cells expressing the MC4 receptor at a 10 μM concentration, demonstrating the lack of functional agonist activity of these compounds. Instead, these compounds showed dose-dependent inhibition of α-MSH-stimulated cAMP production. For example, **11** and **12e** had IC_{50} values of 210 and 510 nM, respectively, in this assay.

Compounds **11**, **12a**, and **12e** were profiled for their pharmacokinetic properties in mice (Table 4). After an intravenous injection at 5 mg/kg, the moderately lipophilic piperazinone **11** (measured log *D* = 2.8) exhibited a moderate plasma clearance (CL = 20 mL/min kg) and a high volume of distribution (V_d = 13 L/kg), which resulted in a long half-life ($t_{1/2}$ = 7.7 h) in this species. The high V_d might be associated with its dibasic nature, although the piperazinone moiety might only be weakly basic. The whole brain concentration was only about 40 ng/g at 1 h post-dosing, and the brain-to-plasma ratio judged by area under curve (AUC) values was 32%. In comparison, the lipophilic pyrrolidinone **12a** (measured log *D* of 3.5 vs 2.8 for **11**) had a low plasma CL of 5.1 mL/min kg, a low V_d of 1.8 L/kg, and a moderate $t_{1/2}$ of 4.1 h presumably due to its high plasma protein binding.[19] Another pyrrolidinone **12e** (measured log *D* = 3.1) had a moderate plasma CL of 31 mL/min kg, a moderate V_d of 5.8 L/kg, although its $t_{1/2}$ of 2.2 h was short in this species. At the 1- and 4-h time points post-dosing, the whole brain concentrations were 484 and 154 ng/g, resulting in brain/plasma ratios of 1.27 and 0.81, respectively.

Table 3. Selectivity profiles of MC4R ligands

Compound	K_i[a] (nM)			
	MC1R	MC3R	MC4R	MC5R
S-**10c**	n.d.	450	0.8	240
11	(23%)	950	4.5	360
12a	(21%)	640	4.5	250
12e	(31%)	1,800	11	990

[a] Data are average of two or more independent measurements.

Table 4. Pharmacokinetic parameters of compounds **10**, **12a**, and **12e** in mice[a]

Compound	**10**	**12a**	**12e**
iv dose (mg/kg)	5	5	5
CL (mL/min kg)	20	5.1	31
V_d (L/kg)	13	1.8	5.8
$t_{1/2}$ (h)	7.7	4.1	2.2
AUC (ng/mL h)	3943	16,300	2678
C_{brain} (ng/g)@1, 4 h	41, 46[b]	397, 115	484, 154
C_{brain}/C_{plasma}	0.07, 0.24[c]	0.12, 0.13	1.27, 0.81
po dose (mg/kg)	10	10	10
C_{max} (ng/mL)	214	560	270
T_{max} (h)	0.5	0.5	0.5
AUC (ng/ml h)	1527	1400	2271
F (%)	19.4	4.3	42.4

[a] Data are average of three animals.
[b] Brain AUC (0–24 h): 1262 ng/g h.
[c] Ratio of 0.32 based on AUCs.

After an oral dose of 10 mg/kg, **11** reached a maximal concentration of 214 ng/mL at 0.5 h to give an AUC of 1527 ng/mL h, which resulted in an absolute bioavailability of 19.4%. For **12a**, although it displayed high plasma concentrations (C_{max} = 560 ng/mL, AUC = 1400 ng/mL h), its absolute oral bioavailability was only 4.3%. In comparison, **12e** reached a maximal plasma concentration of 270 ng/mL at 0.5 h to give an AUC of 2271 ng/mL h, resulting in a high absolute bioavailability of 42.4% (Fig. 2).

Compound **12e** displayed favorable pharmacokinetic properties and therefore it was further studied in a mouse cancer cachexia model as previously described.[3a] It was found that **12e** possessed high binding affinity (K_i) of 5.5 nM at the mouse MC4 receptor in vitro (9.1 nM, rat MC4R). For the in vivo study, C57BL/6J male mice were inoculated with Lewis lung carcinoma (LLC) tumor cells. Beginning 12 days after LLC inoculation, animals were treated over 4 days with **12e** twice daily (3 or 9 mg/kg ip). LLC tumor bearing mice treated with **12e** showed a significant increase in food intake relative to vehicle-treated tumor bearing controls (Fig. 3a). Body weight was also significantly increased in mice treated with **12e** (Fig. 3b). Analysis of body composition with dual-energy X-ray absorptiometry (DEXA) demonstrated that the greater increase of body weight in **12e** treated mice was due to sparing of lean mass as well as body fat (Fig. 4a and b). Tumor bearing vehicle treated animals demonstrated only a 3% increase in LBM over the course of the 15-day experiment, whereas tumor-bearing animals treated with **12e** increased their LBM by ~20%. Fat mass actually decreased by ~3% in vehicle treated animals but increased by ~10% in mice treated with the MC4 antagonist. The difference between the 3 and 9 mg/kg groups was not statistically significance. It is worth noting that **12e** was a very weak

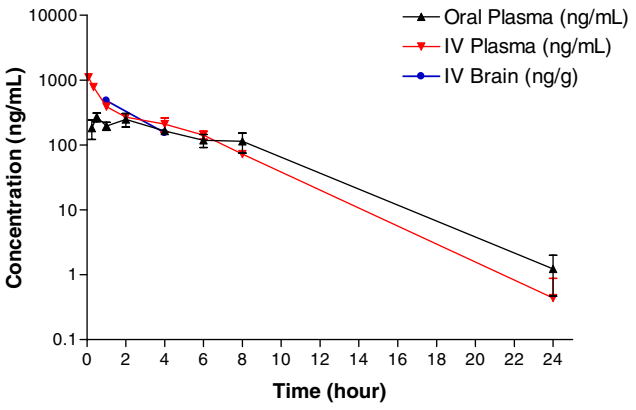


Figure 2. Time-concentration curve of **12e** in mice after 5 mg/kg intravenous injection and 10 mg/kg oral gavage administration.

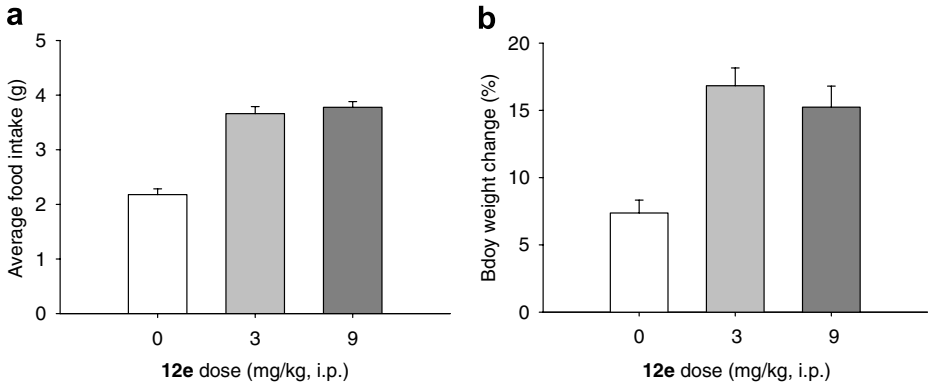


Figure 3. Food intake and body weight changes in LLC tumor-bearing mice in comparison with controls after administration of compound **12e**.

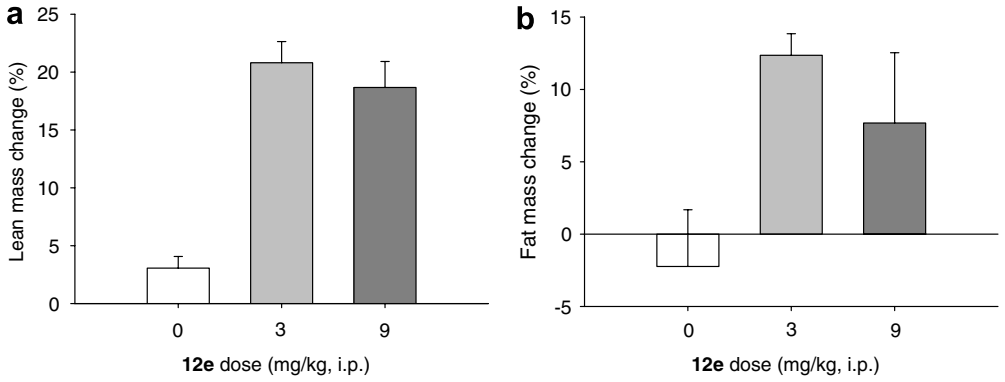


Figure 4. Lean and fat mass changes in LLC tumor-bearing mice in comparison with controls after administration of compound **12e**.

partial ghrelin agonist, with an EC_{50} value of 4.1 μM (47% intrinsic activity) in an in vitro assay, therefore, the contribution of the ghrelin component to the observed efficacy should be minimal.[20]

In conclusion, a series of phenylalaninepiperazine derivatives were synthesized as potent and selective antagonists of the melanocortin-4 receptor. In addition, several compounds were profiled for their pharmacokinetic properties in mice. Compound **12e** was found to have favorable pharmacokinetics and demonstrated to be efficacious in a mouse cachexia model.

1. Experimental

1.1. Chemistry

1.1.1. General methods. NMR spectra were recorded on a Varian 300 MHz spectrometer with TMS as an internal standard, and ^{13}C NMR spectra were recorded at 75 MHz. 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 220 and 254 nm).

1.1.2. 1-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-amino-3-(2,4-dichlorophenyl)propionyl]}-piperazine (6). To a solution of 4-{2-[(1*S*)-((*S*)-2-methylpropanesulfinylamino)-3-methylbutyl]-4-trifluoromethylphenyl}-piperazine-1-carboxylic acid *tert*-butyl ester (Boc-*S*-**4d**,[7] 5.0 g, 9.6 mmol) in CH_2Cl_2 (80 mL) was added TFA (20 mL). The reaction mixture was stirred at room temperature for 1 h, quenched with saturated $NaHCO_3$ aqueous solution, and extracted with ethyl acetate (3 × 100 mL). The combined organic layers were dried over Na_2SO_4. The solvents were removed in vacuo. The residue was dissolved in 20 mL of DMF/DCM (1:3) followed by the addition of *N*-Boc-D(2,4-Cl)Phe-OH (3.54 g, 10.6 mmol), HOBt (1.95 g, 14.4 mmol), EDC (2.77 g, 14.4 mmol), and $NaHCO_3$ (2.42 g, 28.9 mmol). The reaction mixture was stirred at room temperature overnight, diluted with 100 mL of ethyl acetate, and washed with 1 N HCl aqueous solution, saturated $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4, and the solvents were removed in vacuo. The product was purified using flash column chromatography on silica gel (30–40% ethyl acetate in hexanes) to give 1-{4-[2-(1*S*)-((*S*)-2-methylpropanesulfinylamino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-*tert*-butoxycarbonylamino-3-(2,4-dichlorophenyl)propionyl]}-piperazine (Boc-*S*-**5d**) as a white foam (5.24 g, 74% yield).

The above white foam (30 mg, 0.04 mmol) was dissolved in 5 mL of MeOH and treated with 4 N HCl in 1,4-dioxane

(27 μL, 0.11 mmol). The reaction mixture was stirred at room temperature for 1 h. MeOH and excess of HCl were removed in vacuo, and the residue was treated with 5 mL of 30% TFA in CH_2Cl_2. After stirring for 1 h, excess of TFA and CH_2Cl_2 were removed in vacuo, and the product was purified using flash silica chromatograph column (10–15% MeOH/CH_2Cl_2) to give the titled compound as colorless oil (15 mg). HPLC purity: 97.8% (220 nm), 95% (254 nn). ^{1}H NMR (DMSO-d_6): 7.82 (d, J = 1.8 Hz, 1H), 7.59 (d, J = 1.3 Hz, 1H), 7.48 (dd, J = 1.8, 8.3 Hz, 1H), 7.30–7.38 (m, 2H), 7.12 (d, J = 8.3 Hz, 1H), 4.28–4.36 (m, 1H), 3.96–4.06 (m, 1H), 3.04–3.64 (m, 8H), 2.92–2.02 (m, 1H), 2.80–2.86 (m, 1H), 1.10–1.60 (m, 3H), 0.86 (d, J = 6.4 Hz, 3H), 0.84 (d, J = 6.4 Hz, 3H); ^{13}C NMR: 173.6, 153.9, 144.7, 135.8, 135.0, 134.3, 132.6, 129.2, 127.8, 125.5 (q, J = 31.3 Hz), 125.2 (q, J = 271.6 Hz), 124.7, 124.3, 121.3, 53.0, 49.9 (2C), 49.6, 47.3, 45.9, 42.5, 25.3, 23.9, 22.8 (2C); HRMS (MH^+) calcd for $C_{25}H_{31}Cl_2F_3N_4O$ 531.1900, found 531.1875.

1.1.3. 1-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-acetamido-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*S*-7a). To a solution of 1-{4-[2-(1*S*)-((*S*)-2-methylpropanesulfinylamino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-*tert*-butoxycarbonylamino-3-(2,4-dichlorophenyl)propionyl]}-piperazine (Boc-*S*-**5d**, 1.47 g, 2.8 mmol) in CH_2Cl_2 (18 mL) was added TFA (2 mL). The reaction mixture was stirred at room temperature for 1 h, quenched with saturated $NaHCO_3$ aqueous solution, and extracted with ethyl acetate (3 × 50 mL). The combined organic layers were dried over Na_2SO_4 and solvents were removed in vacuo to give 1-{4-[(1*S*)-2-((*S*)-2-methylpropanesulfinylamino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-amino-3-(2,4-dichlorophenyl)propionyl]}-piperazine *S*-**5d** without further purification.

To a solution of *S*-**5d** (50 mg) in CH_2Cl_2 (2 mL) was added acetic anhydride (1 mL, excess). The mixture was stirred at room temperature for 1 h and then concentrated in vacuo. The crude product was then dissolved in 2 mL of MeOH and treated with 4 N HCl in 1,4-dioxane (27 μL, 0.11 mmol). The reaction mixture was stirred at room temperature for 1 h. MeOH and excess of HCl were removed in vacuo, and the product was purified using flash column chromatography on silica gel (30–40% ethyl acetate in hexanes) to give the titled compound as colorless oil, HPLC purity: 100% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.43 (d, J = 8.3 Hz, 1H, NH), 7.81 (d, J = 1.3 Hz, 1H), 7.60 (d, J = 1.7 Hz, 1H), 7.48 (dd, J = 1.7, 8.3 Hz, 1H), 7.32–7.40 (m, 2H), 7.12 (d, J = 8.3 Hz, 1H), 5.07 (dd, J = 7.9 Hz, 1H), 4.28–4.36 (m, 1H), 3.10–3.70 (m, 6H), 2.98–3.08 (m, 1H), 2.86–2.95 (m, 1H), 2.60–2.74 (m, 2H), 1.74 (s, 3H), 1.14–1.60 (m, 3H), 0.86 (d, J = 6.4 Hz, 3H), 0.84 (d, J = 6.4 Hz, 3H); ^{13}C NMR: 169.8, 169.4, 153.7, 144.9, 135.1, 134.8, 134.1, 132.9, 129.3, 127.8, 125.5 (q, J = 31.7 Hz), 125.2 (q, J = 271.6 Hz), 124.7, 124.5, 121.3, 64.2, 53.0, 49.7 (2C), 47.7, 47.3, 46.2, 42.7, 35.9, 25.3, 23.9, 23.3, 22.8 (2C); HRMS (MH^+) calcd for $C_{27}H_{33}Cl_2F_3N_4O_2$ 573.2005, found 573.1998.

Compounds *R*-**8a**, *R*-**9a**, *R*-**10a**, and *S*-**10a** were synthesized using a procedure similar to that for *S*-**7a** from the corresponding *R*-**5c**, *R*-**5d**, *R*-**5a**, and *S*-**5a**, respectively.[10]

1.1.4. 1-{4-[(1*R*)-2-(1-Amino-3-methylbutyl)-4-fluorophenyl]-4-[(2*R*)-acetamido-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*R*-8a). White foam, HPLC purity: 100% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.42 (d, J = 8.8 Hz, 1H), 7.55–7.58 (m, 1H), 7.28–7.40 (m, 2H), 7.26 (dd, J = 2.6, 9.6 Hz, 1H), 6.98–7.10 (m, 1H), 6.95 (dd, J = 3.0, 8.3 Hz, 1H), 5.00–5.12 (m, 1H), 4.29–4.38 (m, 1H), 2.80–3.40 (m, 8H), 2.98–3.08 (m, 1H), 2.82–2.94 (m, 1H), 1.76 (s, 3H), 1.14–1.62 (m, 3H), 0.85 (d, J = 6.1 Hz, 3H), 0.84 (d, J = 6.1 Hz, 3H); ^{13}C NMR: 169.8, 169.4, 160.3 (d, J = 240.4 Hz), 147.2, 146.4, 135.1, 134.7 (d, J = 2.8 Hz), 134.1, 133.0, 129.3, 127.9, 123.0 (d, J = 7.8 Hz), 113.9 (2C, d, J = 20.2 Hz), 53, 49.6 (2C), 47.6, 47.3, 46.4, 43, 36.0, 25.3, 23.9, 22.9 (2C); MS: 523 (MH^+).

1.1.5. 1-{4-[(1*R*)-2-(1-Amino-3-methylbutyl)-6-trifluoromethylphenyl]-4-[(2*R*)-acetamido-3-(2,4-dichlorophenyl)-propionyl]}-piperazine (*R*-9a). Colorless foam, HPLC purity: 100% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.36-8.44 (m, 1H), 7.75–7.84 (m, 1H), 7.50–7.60 (m, 2H), 7.30–7.44 (m, 3H), 4.98 (m, 1H), 4.00–4.20 (m, 1H), 3.76–3.92 (m, 1H), 2.8–3.42 (m, 9H), 1.72 (s, 3H), 1.14–1.62 (m, 3H), 0.87 (d, J = 6.1 Hz, 3H), 0.85 (d, J = 6.1 Hz, 3H); ^{13}C NMR: 169.7, 169.4, 151.8, 146.3, 146.2, 134.9, 133.9, 133.4, 132.9 (q, J = 7.8 Hz), 130.3 (q, J =28.4 Hz), 129.2, 127.8 (2 C), 125.9, 125.0 (q, J = 272.1 Hz), 52.3, 51.5 (2C), 47.9, 47.7, 46.6, 43.0, 35.9, 25.1, 24.5, 22.9, 22.3; MS: 574 (MH^+).

1.1.6. 1-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-6-fluorophenyl]-4-[(2*R*)-acetamido-3-(2,4-dichlorophenyl)propionyl]}-piperazine trifluoroacetate (*S*-10a). Purified on HPLC, white solid, HPLC purity: 100% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.41-8.50 (b, 1H), 7.56 (dd, J = 1.8, 4.8 Hz, 1H), 7.26–7.44 (m, 4H), 7.15 (dd d, $J_{H,F}$ = 12.3 Hz, J = 2.6, 7.9 Hz, 1H), 5.00–5.14 (m, 1H), 4.76–4.84 (m, 1H), 4.30–4.46 (m, 1H), 3.86–4.04 (m, 1H), 3.20–3.42 (m, 4H), 2.60–3.18 (m, 4H), 1.74 (s, 3H), 1.58–1.72 (m, 1H), 1.40–1.57 (m, 2H), 0.89 (d, J = 6.4 Hz, 3H), 0.88 (d, J = 6.4 Hz, 3H); ^{13}C NMR: 169.8, 169.4, 162.0 (d, J = 250.5 Hz), 142.3, 136.5, 134.9, 134.5, 134.0, 132.9, 129.2, 128.7, 127.8, 123.4, 116.7, 51.8, 51.0, 47.5, 46.6, 45.7, 43.0, 36.0, 26.4, 24.9, 23.2, 23.1, 22.8; MS: 523 (MH^+).

1.1.7. 1-{4-[(1*R*)-2-(1-Amino-3-methylbutyl)-6-fluorophenyl]-4-[(2*R*)-acetamido-3-(2,4-dichlorophenyl)propionyl]}-piperazine trifluoroacetate (*R*-10a). Purified on HPLC, white solid, HPLC purity: 100% (220 nm). ^{1}H NMR (DMSO-d_6): 8.42-8.50 (b, 1H), 7.56 (dd, J = 1.8, 3.5 Hz, 1H), 7.30–7.42 (m, 4H), 7.18–7.28 (m, 1H), 5.01–5.13 (m, 1H), 4.86–4.98 (m, 1H), 4.32–4.46 (m, 1H), 3.82–4.04 (m, 1H), 2.83–3.20 (m, 6H), 2.60–2.80 (m, 2H), 1.75 (s, 3H), 1.64–1.76 (m, 1H), 1.48–1.62 (m, 1H), 1.30–1.48 (m, 1H), 0.89 (d, J = 6.6 Hz, 3H), 0.87 (d, J = 6.6 Hz, 3H); MS: 523 (MH^+).

1.1.8. 1-{4-[(1*S*)-2-(Amino-3-methylbutyl)-6-fluorophenyl]-4-[(2*R*)-(3-aminopropionylamido)-3-(2,4-dichlorophenyl)-propionyl]}-piperazine (*S*-10c). To a solution of 4-{2-[(1*S*)-((*S*)-2-methylpropanesulfinylamino)-3-methylbutyl]-6-fluorophenyl}-piperazine-1-carboxylic acid *tert*-butyl ester (Boc-*S*-**4a**,[7] 43.0 mg, 0.092 mmol) in 2 mL of CH_2Cl_2 was added 0.5 mL of TFA. The reaction mixture was stirred for 0.5 h, basified with saturated aqueous $NaHCO_3$, and extracted with EtOAc (3×10 mL). After removal of solvents, the residue was dissolved in 2 mL of DMF/CH_2Cl_2 (1:4) followed by the addition of *N*-Boc-β-Ala-(2,4-Cl)Phe-OH (44.5 mg, 0.11 mmol), $NaHCO_3$ (23.1 mg, 0.28 mmol), HOBt (24.8 mg, 0.18 mmol), and EDCI (35.1 mg, 0.18 mmol) sequentially. The reaction mixture was stirred overnight. EtOAc/saturated aqueous $NaHCO_3$ (50 mL/20 mL) workup followed by flash silica chromatograph column (50–60% EtOAc/hexanes) purification gave the product as white foam (54.8 mg, 79%). This white foam was dissolved in 5 mL of MeOH and treated with 4 N HCl in 1,4-dioxane (27 μL, 0.1085 mmol). The reaction mixture was stirred at room temperature for 1 h. MeOH and excess of HCl were removed in vacuo to give white foam which was treated with 5 mL of 30%TFA in CH_2Cl_2. After stirring for 1 h, excess of TFA and CH_2Cl_2 were removed in vacuo and the product was purified with flash silica chromatograph column (10–15% MeOH/CH_2Cl_2) to give the titled compound as oil (34.0 mg, 85%), HPLC purity: 100% (220 nm). ^{1}H NMR (DMSO-d_6): 8.6 (m, 1H), 7.5 (br s, 1H), 7.24–7.38 (m, 3H), 7.10–7.22 (m, 1H), 6.91–7.00 (m, 1H), 5.04–5.16 (m, 1H), 4.28–4.48 (m, 2H), 3.84–3.98 (m, 1H), 2.80–3.20 (m, 6H), 2.56–2.80 (m, 4H), 2.10–2.34 (m, 2H), 1.42–1.70 (m, 1H), 1.18–.44 (m, 2H), 0.80–0.92 (m, 6H) ^{13}C NMR: 171.2, 169.9, 161.9 (d, J = 249.4 Hz), 149.5 (d, J = 3.4 Hz), 135.7 (d, J = 10.3 Hz), 135.0, 134.5, 134.0, 133.1, 129.2 (d, J = 6.1 Hz), 128.0, 127.9, 123.1, 114.7 (d, J = 21.2 Hz), 52.0, 49.2, 48.2, 47.8, 43.1, 38.3, 38.2, 38.0, 36.1, 35.6, 25.3, 24.0, 22.9. HRMS (MH^+): calcd for $C_{27}H_{36}Cl_2FN_5O_2$ 552.2303, found 552.2309.

Compounds *S*-**7c**, *R*-**7c**, *S*-**9c**, *R*-**9c**, and *R*-**10c** were synthesized using a procedure similar to that of *S*-**10c** from the corresponding *S*-**4d**, *R*-**4d**, *S*-**4b**, *R*-**4b**, and *R*-**4a**, respectively.[10]

1.1.9. 1-{4-[(1*S*)-2-(Amino-3-methylbutyl)-4-fluorophenyl]-4-[(2*R*)-(3-aminopropionylamido)-3-(2,4-dichlorophenyl)-propionyl]}-piperazine (*S*-7c). White foam, HPLC purity: 99% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.63 (d, J = 8.3 Hz, 1H), 7.82 (d, J = 1.8 Hz, 1H), 7.61 (d, J = 1.7 Hz, 1H), 7.49 (dd, J =1.8, 8.3 Hz, 1H), 7.30–7.38 (m, 2H), 7.14 (d, J = 8.3 Hz, 1H), 5.02–5.16 (m, 1H), 4.26–4.34 (m, 1H), 3.40–3.64 (m, 4H), 2.80–3.26 (m, 6H), 2.58–2.80 (m, 2H), 2.12–2.18 (m, 2H), 1.32–1.61 (m, 2H), 1.20–1.32 (m, 1H), 0.86 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.6 Hz, 3H); MS: 602 (MH^+).

1.1.10. 1-{4-[(1*R*)-2-(Amino-3-methylbutyl)-4-fluorophenyl]-4-[(2*R*)-(3-aminopropionylamido)-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*R*-7c). White foam, HPLC purity: 91% (220 nm), 92% (254 nm). ^{1}H NMR (DMSO-d_6): 8.53 (d, J = 8.3 Hz, 1H), 7.81 (d, J = 1.8 Hz, 1H), 7.56 (d, J = 1.8 Hz, 1H), 7.47 (dd, J = 1.8, 8.3 Hz, 1H), 7.28–7.38 (m, 2H), 7.13 (d, J = 8.3 Hz, 1H), 5.04–5.15 (m, 1H), 4.26–4.33 (m, 1H), 3.20–3.68 (m, 2H), 2.78–3.28 (m, 8H), 2.56–2.74 (m, 2H), 2.10–2.20 (m, 2H), 1.46–1.61 (m, 1H), 1.32–1.46 (m, 1H), 1.18–1.32 (m, 1H), 0.86 (d, J = 6.6 Hz, 3H), 0.84 (d, J = 6.6 Hz, 3H); MS: 602 (MH^+).

1.1.11. 1-{4-[(1*S*)-2-(Amino-3-methylbutyl)-6-trifluoromethylphenyl]-4-[(2*R*)-(3-aminopropionylamido)-3-(2,4-dichlorophenyl)propionyl]}-piperazine trifluoroacetate (*S*-9c). Purified on HPLC, white powder, HPLC purity: 97% (220 nm), 100% (254 nm). ^{1}H NMR (DMSO-d_6): 8.64–8.76 (b, 1H), 7.82–7.90 (m, 1H), 7.73–7.80 (m, 1H), 7.54–7.68 (m, 2H), 7.29–7.42 (m, 2H), 5.04–5.20 (m, 1H), 4.48–4.68 (m, 1H), 3.64–3.82 (m, 2H), 3.50–3.63 (m, 2H), 3.32–3.46 (m, 2H), 2.75–3.20 (m, 6H), 2.36–2.46 (m, 2H), 1.46–1.96 (m, 1H), 1.59–1.75 (m, 1H), 1.42–1.59 (m, 1H), 0.90 (d, J = 6.1 Hz, 3H), 0.87 (d, J = 6.1 Hz, 3H); MS: 602 (MH^+).

1.1.12. 1-{4-[(1*R*)-2-(Amino-3-methylbutyl)-6-trifluoromethylphenyl]-4-[(2*R*)-(3-aminopropionylamido)-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*R*-9c). Colorless oil, HPLC purity: 100% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.59 (d, J = 8.8 Hz, 1H), 7.74–7.90 (m, 1H), 7.48–7.62 (m, 2H), 7.26–7.46 (m, 3H), 5.00–5.20 (m, 1H), 4.00–4.24 (m, 1H), 3.70–3.94 (m, 1H), 3.20–3.70 (m, 6H), 3.00–3.20 (m, 2H), 2.80–3.00 (m, 2H), 2.70–2.78 (m, 1H), 2.14–2.38 (m, 2H), 1.46–1.61 (m, 1H), 1.32–1.46 (m, 1H), 1.18–1.32 (m, 1H), 0.87 (d, J = 6.6 Hz, 3H), 0.85 (d, J = 6.6 Hz, 3H); MS: 602 (MH^+).

1.1.13. 1-{4-[(1*R*)-2-(Amino-3-methylbutyl)-6-fluorophenyl]-4-[(2*R*)-(3-aminopropionylamido)-3-(2,4-dichlorophenyl)propionyl]}-piperazine trifluoroacetate (*R*-10c). Purified on HPLC, white powder, HPLC purity: 100% (220 nm). ^{1}H NMR (DMSO-d_6): 8.67–8.77 (b, 1H), 7.58 (dd, J = 2.2, 4.3 Hz, 1H), 7.28–7.42 (m, 4H), 7.22 (dd d, $J_{H,F}$ = 12.3 Hz, J = 2.2, 7.5 Hz, 1H), 5.05–5.18 (m, 1H), 4.88–4.97 (m, 1H), 4.32–4.45 (m, 1H), 3.79–4.02 (m, 1H), 2.58–3.20 (m, 10H), 2.39–2.48 (m, 2H), 1.65–1.78 (m, 1H), 1.47–1.62 (m, 1H), 1.32–1.46 (m, 1H), 0.88 (d, J = 6.1 Hz, 6H); MS: 552 (MH^+).

1.1.14. 1-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-aminoacetamido-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*S*-7b). This compound was prepared using a procedure similar to that of *S*-**10c**, but replacing *N*-Boc-Ala-D(2,4-Cl)Phe-OH with *N*-Boc-Gly-D(2,4-Cl)Phe-OH.

White foam, HPLC purity: 99% (220 nm), 100% (254 nm). ^{1}H NMR (DMSO-d_6): 8.26–8.38 (b, 1H, NH), 7.80–7.84 (br s, 1H), 7.60 (d, J = 1.8 Hz, 1H), 7.46 (dd, J = 1.8, 8.8 Hz, 1H), 7.28–7.40 (m, 2H), 7.13 (d, J = 8.3 Hz, 1H), 5.06–5.20 (m, 1H), 4.26–4.38 (m, 1H), 3.40–3.64 (m, 2H), 2.80–3.40 (m, 6H), 2.20–2.36 (m, 2H), 2.60–2.70 (m, 2H), 1.84–1.90 (m, 1H), 1.14–1.62 (m, 2H), 0.86 (d, J = 6.1 Hz, 3H), 0.84 (d, J = 6.1 Hz, 3H); ^{13}C NMR: 169.5 (2C), 153.7, 144.9,

135.1, 134.5, 134.2, 133.0, 129.3, 127.9, 125.5 (q, J = 31.3 Hz), 125.2 (q, J = 271.6 Hz), 124.7, 124.3, 121.3, 64.2, 53.0, 47.4, 47.3, 46.2, 42.7, 36.1, 29.7, 25.3, 23.9, 22.8, 18.6; HRMS (MH^+) calcd for $C_{27}H_{34}Cl_2F_3N_5O_2$ 588.2114, found 588.2101.

Compounds *S*-**9b**, *R*-**9b**, *S*-**10b**, and *R*-**10b** were synthesized using a procedure similar to that of *S*-**7b** from *S*-**4b**, *R*-**4b**, *S*-**4a**, and *R*-**4a**, respectively.[10]

1.1.15. 1-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-6-trifluoromethylphenyl]-4-[(2*R*)-aminoacetamido-3-(2,4-dichlorophenyl)propionyl]}-piperazine trifluoroacetate (*S*-9b). Purified on HPLC, white powder, HPLC purity: 95% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.86–9.00 (b, 1H), 7.87 (d, J = 1.8 Hz, 1H), 7.72–7.79 (m, 1H), 7.53–7.63 (m, 2H), 7.28–7.42 (m, 2H), 5.13–5.24 (m, 1H), 4.50–4.67 (m, 1H), 3.67–3.80 (m, 1H), 3.47–3.65 (m, 3H), 3.32–3.46 (m, 1H), 3.04–3.16 (m, 2H), 2.81–3.03 (m, 4H), 2.70–2.80 (m, 1H), 1.79–2.00 (m, 1H), 1.59–1.78 (m, 1H), 1.45–1.59 (m, 1H), 0.89 (d, J = 6.5 Hz, 3H), 0.87 (d, J = 6.5 Hz, 3H); MS: 588 (MH^+).

1.1.16. 1-{4-[(1*R*)-2-(1-Amino-3-methylbutyl)-6-trifluoromethylphenyl]-4-[(2*R*)-aminoacetamido-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*R*-9b). White solid, HPLC purity: 100% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.30–8.42 (b, 1H, NH), 7.76–7.90 (m, 1H), 7.50–7.58 (m, 2H), 7.22–7.46 (m, 3H), 5.06–5.20 (m, 1H), 4.06–4.20 (m, 1H), 3.74–3.94 (m, 2H), 3.40–3.64 (m, 2H), 2.80–3.40 (m, 6H), 3.00–3.16 (m, 1H), 2.84–2.98 (m, 1H), 1.80–1.90 (m, 1H), 1.14–1.62 (m, 2H), 0.87 (d, J = 6.2 Hz, 3H), 0.85 (d, J = 6.2 Hz, 3H); MS: 588 (MH^+).

1.1.17. 1-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-6-fluorophenyl]-4-[(2*R*)-aminoacetamido-3-(2,4-dichlorophenyl)-propionyl]}-piperazine trifluoroacetate (*S*-10b). Purified on HPLC, white solid, HPLC purity: 100% (220 nm). ^{1}H NMR (DMSO-d_6): 8.60–8.72 (b, 1H), 7.57 (dd, J = 1.8, 6.1 Hz, 1H), 7.28–7.42 (m, 4H), 7.11–7.21 (m, 1H), 5.10–5.21 (m, 1H), 4.81–4.90 (m, 1H), 4.31–4.45 (m, 1H), 3.86–4.03 (m, 1H), 3.22–3.52 (m, 6H), 2.88–3.10 (m, 2H), 2.58–2.83 (m, 2H), 1.60–1.73 (m, 1H), 1.36–1.56 (m, 2H), 0.86 (d, J = 6.1 Hz, 6H); MS: 538 (MH^+).

1.1.18. 1-{4-[(1*R*)-2-(1-Amino-3-methylbutyl)-6-fluorophenyl]-4-[(2*R*)-aminoacetamido-3-(2,4-dichlorophenyl)-propionyl]}-piperazine trifluoroacetate (*R*-9b). Purified on HPLC, white powder, HPLC purity: 100% (220 nm). ^{1}H NMR (DMSO-d_6): 8.96 (d, J = 8.3 Hz, 1H), 7.57–7.62 (m, 1H), 7.28–7.43 (m, 4H), 7.22 (dd d, $J_{H,F}$ = 12.3 Hz, J = 2.2, 7.5 Hz, 1H), 5.12–5.24 (m, 1H), 4.85–4.98 (m, 1H), 4.36–4.46 (m, 1H), 3.80–4.03 (m, 1H), 3.44–3.60 (m, 2H), 2.60–3.14 (m, 8H), 1.64–1.78 (m, 1H), 1.46–1.62 (m, 1H), 1.32–1.46 (m, 1H), 0.88 (d, J = 6.1 Hz, 6H); MS: 538 (MH^+).

1.1.19. 1-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-(piperidin-4-ylcarboxamido)-3-(2,4-dichlorophenyl)propionyl]}-piperazine trifluoroacetate (*S*-7d). To a solution of 1-{4-[(1*S*)-2-((*S*)-2-methylpropanesulfinylamino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-amino-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*S*-**5d**, 50 mg, 0.079 mmol) in DMF/CH_2Cl_2 (2 mL) was added *N*-Boc-isonipecotic acid (23 mg, 0.1 mmol), followed by HOBt (20 mg, 0.14 mmol), EDCI (28 mg, 0.14 mmol), and $NaHCO_3$ (24 mg, 0.3 mmol). The mixture was stirred at rt overnight and then concentrated in vacuo. The crude product was then dissolved in 5 mL of MeOH and treated with 4 N HCl in 1,4-dioxane (27 μL, 0.1085 mmol), stirred at rt for 1 h, and concentrated in vacuo, and the residue was treated with 30% TFA/CH_2Cl_2 at rt for 1 h. The product was then purified using HPLC and obtained as white powder, HPLC purity: 100% (220 and 254 nm). ^{1}H NMR (DMSO-d_6): 8.49 (d, J = 8.3 Hz, 1H), 7.93 (d, J = 1.3 Hz, 1H), 7.68 (dd, J = 1.3, 8.8 Hz, 1H), 7.59 (s, 1H), 7.35 (br s, 2H), 7.33 (d, J = 8.8 Hz, 1H), 5.03–5.14 (m, 1H), 4.70–4.80 (m, 1H), 4.04–4.16 (m, 1H), 3.12–3.40 (m, 9H), 3.02–3.12 (m, 1H), 2.90–3.00 (m, 1H), 2.74–2.89 (m, 2H), 2.56–2.70 (m, 1H), 1.62–1.80 (m, 3H), 1.30–1.62 (m, 3H), 0.90 (d, J = 6.6 Hz, 3H), 0.83 (d, J = 6.6 Hz, 3H); ^{13}C NMR: 173.3, 169.7, 154.7, 137.4, 135.1, 134.7, 134.3, 132.9, 129.2, 127.7, 126.7 (q, J = 5.6 Hz), 126.3 (q, J = 32.2 Hz), 125.0 (q, J = 271.7 Hz), 124.9, 122.9, 53.0, 49.2, 47.6, 47.0, 46.0, 45.8, 43.0 (2C), 42.6, 39.2, 35.7, 25.9, 25.6, 24.7, 23.0 (2C); HRMS (MH^+) calcd for $C_{31}H_{40}Cl_2F_3N_5O_2$ 642.2584, found 642.2583.

1.1.20. 1-[(*R*)-2-{4-[(1*S*)-2-(1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]piperazin-1-yl}-1-(2,4-dichlorobenzyl)-2-oxoethyl]-piperazin-2-one dihydrochloride (11). A solution of 1-{4-[(1*S*)-2-((*S*)-2-methylpropanesulfinylamino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-amino-3-(2,4-dichlorophenyl)propionyl]}-piperazine (*S*-**5d**, 739 mg, 1.2 mmol) in CH_2Cl_2 (12 mL) was treated with glacial acetic acid (1 drop) and *tert*-butyl-*N*-(2-oxoethyl)carbamate (372 mg, 2.34 mmol). Stirring was continued at ambient temperature for 0.5 h, and then $Na(OAc)_3BH$ (1.24 g, 5.85 mmol) was added portionwise. The resulting suspension was stirred at room temperature for 14 h, after which time the reaction was deemed complete by LCMS. The reaction mixture was transferred to a separatory funnel, diluted with CH_2Cl_2 (50 mL), and washed with saturated $NaHCO_3$ aqueous solution (2 × 50 mL). The organic layers were dried over anhydrous $MgSO_4$, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel, eluting with ethyl acetate, and the product was obtained as white foam (534 mg, 59% yield).

The above compound (368 mg, 0.47 mmol) was dissolved in ethyl acetate (3 mL) and treated with saturated $NaHCO_3$ aqueous solution (3 mL). The resulting biphasic system was stirred vigorously and chloroacetyl chloride (57 μL, 0.71 mmol) was added dropwise. After 1 h the reaction was complete according to TLC analysis (EtOAc). The organic layer was then separated and evaporated to give a crude product as white foam (340 mg, 84%). This foam was dissolved in CH_2Cl_2 (5 mL) and treated with HCl (360 μL of a 4.0 M solution

in 1,4-dioxane, 1.42 mmol). After 2 h at room temperature, the volatiles were removed in vacuo and the crude residue was purified by preparative TLC plate (500 μm), eluting with a 400:50:2 v/v mixture of $CHCl_3$, MeOH, and NH_4OH, respectively. The free base (39 mg, 0.06 mmol, 13%) was converted to the respective hydrochloride salt, by treatment with an HCl solution in diethyl ether. The salt was obtained as a white solid (45 mg).

Free base: ^{1}H NMR ($CDCl_3$): 7.65 (d, J = 1.8 Hz, 1H), 7.45 (dd, J_1 = 1.8 Hz, J_2 = 8.1 Hz, 1H), 7.42 (d, J = 1.5 Hz, 1H), 7.26–7.18 (m, 2H), 7.02 (d, J = 9.0 Hz, 1H), 5.99 (t, J = 7.7 Hz, 1H), 4.50 (t, J = 7.2 Hz, 1H), 3.72–3.57 (br s, 3H), 3.54–3.47 (m, 4H), 3.23 (ABX, J_1 = 8.1 Hz, J_2 = 13.5 Hz, $\Delta\nu$ = 46.8 Hz, 2H), 3.09 (t, J = 5.2 Hz, 2H), 2.93–2.89 (m, 1H), 2.66 (br s, 3 H), 1.71 (br s, 2 H), 1.61–1.50 (m, 2H), 1.47–1.40 (m, 1H), 0.92 (dd, J = 4.6 Hz, 6H); MS: 614.5 (MH^+). Anal. calcd for $C_{29}H_{36}Cl_2F_3N_5O_2{\cdot}2HCl{\cdot}CH_2Cl_2$: C 46.65%, H 5.22%, N 9.07%; found: C 46.79%, H 4.98%, N 9.01%.

1.1.21. 1-[(*R*)-2-{4-[2-((*S*)-1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]piperazin-1-yl}-1-(2,4-dichlorobenzyl)-2-oxoethyl]pyrrolidin-2-one mesylate (12a). 1-{4-[(1*S*)-2-((*S*)-2-Methylpropanesulfinylamino-3-methylbutyl)-4-trifluoromethylphenyl]-4-[(2*R*)-amino-3-(2,4-dichlorophenyl)-propionyl]}-piperazine (*S*-**5d**, 2.86 g, 4.5 mmol) was dissolved in 15 mL of 1,2-dichloroethane followed by the addition of succinic semialdehyde (15% in water, 3 mL, 4.8 mmol), HOAc (274 μL, 4.8 mmol), and $NaBH(OAc)_3$ (1.02 g, 4.8 mmol). The reaction mixture was stirred at room temperature overnight, followed by the addition of acetic anhydride (375 μL, 4.8 mmol). The reaction mixture was stirred for another 2 h diluted with 100 mL of EtOAc, washed with saturated $NaHCO_3$ aqueous solution and brine. The organic layer was dried over anhydrous Na_2SO_4, and solvents were removed in vacuo. The residue was purified with flash silica column chromatograph (50% EtOAc in hexanes) to give a sulfinimide as white foam (2.06 g, 65%).

The above intermediate (1.05 g, 1.5 mmol) was dissolved in 50 mL of MeOH and treated with HCl (4 N in 1,4-dioxane, 0.48 mL, 1.92 mmol). The reaction mixture was stirred for 1 h, MeOH and excess HCl were removed in vacuo. The crude product was purified with flash silica column chromatograph (8% MeOH in CH_2Cl_2) to give **12a** as white foam (0.70 g, 78% yield), HPLC purity: 99.3% (220 nm) and 99.1% (254 nm). The free base was converted into a mesylate salt using a standard procedure. ^{1}H NMR ($CDCl_3$): 7.94 (d, $^3J_{H,H}$ = 1.4 Hz, 1H), 7.56 (d, $^3J_{H,H}$ = 1.8 Hz, 1H), 7.67–7.74 (m, 1H), 7.34 (d, $^3J_{H,H}$ = 1.8 Hz, 1H), 7.30–7.42 (m, 2H), 5.20–5.30 (m, 1H), 4.70–4.86 (m, 1H), 3.36–3.64 (m, 5H), 3.16–3.30 (m, 2H), 3.02–3.14 (m, 2H), 2.80–3.02 (m, 2H), 2.60–2.74 (m, 1H), 2.31 (s, 3H, $MeSO_3H$), 2.00–2.14 (m, 2H), 1.82–1.98 (m, 2H), 1.70–1.84 (m, 1H), 1.46–1.60 (m, 1H), 1.26–1.42 (m, 1H), 0.91 (t, $^3J_{H,H}$ = 6.4 Hz, 3H), 0.83 (t, $^3J_{H,H}$ = 6.4 Hz, 3H); ^{13}C NMR: 174.04, 167.55, 154.83, 136.17, 135.10, 134.92, 133.82, 132.77, 129.24, 127.83, 127.04, 126.52, 124.83, 124.55 (q, $^1H_{C,F}$ = 234 Hz), 123.26, 50.06, 47.29, 45.88, 45.27, 43.59, 42.65, 40.36, 39.78, 39.76 ($MeSO_3H$), 39.69, 30.68, 24.66, 23.02, 22.98, 18.56; MS: 599.1 (MH^+); Anal. calcd for $C_{29}H_{35}Cl_2F_3N_4O_2{\cdot}MeSO_3\ H{\cdot}2.5H_2O$: C 48.65%, H 5.99%, N 7.56%; found: C 48.66%, H 5.96%, N, 7.59%.

Compounds **12b**, **12c**, **12d**, and **12f** were synthesized using a procedure similar to that of **12a** from *S*-**4c**, *S*-**4f**, *S*-**4a**, and *S*-**4e**, respectively.[10]

1.1.22. 1-[(*R*)-2-{4-[2-((*S*)-1-Amino-3-methylbutyl)-4-fluorophenyl]piperazin-1-yl}-1-(2,4-dichlorobenzyl)-2-oxoethyl]pyrrolidin-2-one (12b). White foam, HPLC purity: 100% (220 and 254 nm); ^{1}H NMR ($CDCl_3$): 7.35–7.42 (m, 1H), 7.10–7.30 (m, 4H), 6.90–7.01 (m, 1H), 5.38–5.53 (m, 1H), 4.74–4.90 (m, 1H), 3.40–3.64 (m, 2H), 3.22–3.34 (m, 1H), 3.06–3.21 (m, 1H), 2.44–2.90 (m, 3H), 2.20–2.38 (m, 2H), 1.90–2.10 (m, 2H), 1.36–1.90 (m, 5H), 1.18–1.36 (m, 2H), 0.98–1.10 (m, 1H), 0.91 (d, J = 7.0 Hz, 3H), 0.88 (d, J = 7.0 Hz, 3H); MS: 549 (MH^+).

1.1.23. 1-[(*R*)-2-{4-[2-((*S*)-1-Amino-3-methylbutyl)phenyl]-piperazin-1-yl}-1-(2,4-dichlorobenzyl)-2-oxoethyl]pyrrolidin-2-one (12c). White foam, HPLC purity: 90% (220 nm), 97% (254 nm); ^{1}H NMR ($CDCl_3$): 7.35–7.42 (m, 2H), 7.10–7.24 (m, 4H), 6.95 (d, J = 8.3 Hz, 1H), 5.48 (dd, J = 7.4 Hz, 1H), 4.44–4.58 (m, 1H), 3.00–3.84 (m, 6H), 3.22–3.32 (m, 1H), 3.10–3.20 (m, 1H), 2.44–2.90 (m, 3H), 2.20–2.36 (m, 2H), 1.90–2.10 (m, 2H), 1.36–1.62 (m, 3H), 0.92–1.06 (m, 1H), 0.90 (d, J = 6.1 Hz, 3H), 0.88 (d, J = 6.1 Hz, 3H); ^{13}C NMR: 174.7, 167.7, 150.0, 135.5, 133.8, 133.5 (2C), 132.7, 129.7, 128.0, 127.4, 127.0, 125.9, 121.2, 53.7, 49.4, 48.6, 47.6, 46.6, 44.0, 43.0, 40.0, 33.1, 30.9, 25.5, 23.2, 22.7, 18.6; MS: 531 (MH^+).

1.1.24. 1-[(*R*)-2-{4-[2-((*S*)-1-Amino-3-methylbutyl)-6-fluorophenyl]piperazin-1-yl}-1-(2,4-dichlorobenzyl)-2-oxoethyl]pyrrolidin-2-one (12d). White foam, HPLC purity: 100% (220 nm); ^{1}H NMR 300 MHz (DMSO-d_6): 7.54 (br s, 1H), 7.13–7.40 (m, 3H), 6.97 (dd d, $J_{H,F}$ = 12.7 Hz, J = 1.8, 8.3 Hz, 1H), 5.18–5.33 (m, 1H), 4.32–4.46 (m, 1H), 3.71–3.83 (m, 1H), 3.47–3.65 (m, 1H), 2.95–3.40 (m, 6H), 2.60–2.90 (m, 4H), 2.04–2.18 (m, 2H), 1.82–2.00 (m, 2H), 1.52–1.64 (m, 2H), 1.10–1.44 (m, 2H), 0.88 (d, J = 6.1 Hz, 3H), 0.86 (d, J = 6.1 Hz, 3H); MS: 549 (MH^+).

1.1.25. 1-[(*R*)-2-{4-[2-((*S*)-1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]-piperazin-1-yl}-1-(4-dichlorobenzyl)-2-oxoethyl]pyrrolidin-2-one mesylate (12e). A solution of (*R*)-3-(4-chloro-phenyl)-2-(2-oxo-pyrrolidin-1-yl)-propionic acid (*R*-**14b**,[12] 139 mg, 0.52 mmol) in DMF (5 mL) was treated with diisopropylethylamine (180 μL, 1.04 mmol) and HBTU (256 mg, 0.68 mmol) under N_2. The resulting mixture was stirred at room temperature for 0.5 h and then treated with a solution of 4-{4-trifluoromethyl-2-[(*S*)-3-methyl-1-(2-methylpropane-2-sulfinylamino)-butyl]-phenyl}-piperazine (*S*-**4d**, 217 mg, 0.52 mmol), obtained from 4-{4-trifluoromethyl-2-[(*S*)-3-methyl-1-(2-methylpropane-2-sulfinylamino)butyl]-phenyl}-piper-

azine-1-carboxylic acid *tert*-butyl ester (Boc-*S*-**4d**, 292 mg, 0.56 mmol) with TFA treatment as previously described, in DMF (1 mL). The reaction was deemed complete after 2 h at room temperature, monitored by LC/MS. The mixture was diluted with EtOAc (50 mL) and placed in a separatory funnel. The organic layer was washed with 0.1 N HCl aqueous (50 mL), saturated $NaHCO_3$ (100 mL), and brine (50 mL), dried over anhydrous $MgSO_4$, filtered, and evaporated in vacuo to give the crude product as a tan film, which was used without further purification.

The above compound (347 mg, 0.52 mmol) was dissolved in MeOH (5 mL) and treated with HCl (340 μL of a 2.0 M solution in Et_2O, 0.68 mmol). The resulting solution was stirred at ambient temperature for 1 h. The volatiles were removed in vacuo and the residue was purified by preparative HPLC/MS. The free base was isolated in 60% yield (187 mg, 0.33 mmol). HPLC purity: 99.4% (220 nm) and 100% (254 nm). The compound was converted to the mesylate salt in CH_2Cl_2 using one equivalent of methanesulfonic acid.

Free base: ^{1}H NMR (DMSO-d_6): 7.82 (d, J = 1.8 Hz, 1H), 7.49 (dd, J_1 = 1.8 Hz, J_2 = 8.4 Hz, 1H), 7.28 (dd, J_1 = 8.4 Hz, J_2 = 15.9 Hz, 4H), 7.17 (d, J = 8.4 Hz, 1H), 5.13 (t, J = 8.4 Hz, 1H), 4.36 (t, J = 7.5 Hz, 1H), 3.50–3.37 (m, 2H), 3.29–3.21 (m, 4H), 3.06–2.83 (m, 6H), 2.63 (br, 1H), 2.17–2.01 (m, 1H), 1.92–1.78 (m, 2H), 1.55–1.42 (m, 2 H), 1.33–1.21 (m, 1H), 0.84 (dd, J = 6.6 Hz, 6H); ^{13}C NMR: 174.0, 167.9, 153.8, 143.9, 137.3, 131.9, 131.3 (d, J = 29.2 Hz), 128.8, 125.5 (d, J = 31.4 Hz), 125.2 (d, J = 270.3 Hz), 124.7, 121.5, 53.5, 52.8, 51.9, 49.3, 47.2, 45.9, 43.6, 42.7, 34.4, 30.8, 25.2, 23.7, 22.8, 18.6; MS: 565.2 (MH^+). Anal. calcd for $C_{29}H_{36}ClF_3N_4O_2{\cdot}CH_3SO_3$ H·2/3 CH_2Cl_2: C 51.31, H 5.80, N 7.81; found: C 50.97%, H 5.74%, N 7.64%.

1.1.26. 1-[(*R*)-2-{4-[2-((*S*)-1-Amino-3-methylbutyl)phenyl]-piperazin-1-yl}-1-(4-dichlorobenzyl)-2-oxoethyl]pyrrolidin-2-one mesylate (12f). This compound was prepared using a procedure similar to that of **12e** from *S*-**4e**.

Free base as white foam, HPLC purity: 97% (220 nm), 96% (254 nm); ^{1}H NMR ($CDCl_3$): 7.37 (dd, J = 1.8 Hz, J = 7.5 Hz, 1H), 7.08–7.32 (m, 6H), 6.94 (d, J = 7.0 Hz, 1H), 5.31 (dd, J = 7.3 Hz, 1H), 4.44–4.58 (m, 1H), 3.15–3.84 (m, 5H), 3.20–3.33 (m, ABX, 1H), 2.5–2.95 (m, ABX, 1H), 2.66–2.86 (m, 2H), 2.46–2.64 (m, 1H), 1.82–2.40 (m, 6H), 1.40–1.62 (m, 2H), 0.96–1.12 (m, 1H), 0.89 (d, J = 6.1 Hz, 3H), 0.87 (d, J = 6.1 Hz, 3H); ^{13}C NMR: 174.8, 167.7, 150.0, 135.6 (2C), 133.0, 131.1 (2C), 128.9 (2C), 128.0, 126.9, 125.9, 121.3, 53.7, 51.3, 48.6, 47.6, 46.6, 44.1, 43.6, 43.0, 35.3, 31.0, 25.5, 23.2, 22.7, 18.6; HRMS (MH^+) calcd for $C_{28}H_{37}ClN_4O_2$ 497.2678, found 497.2667.

1.1.27. 1-[(*S*)-2-{4-[2-((*S*)-1-Amino-3-methylbutyl)-4-trifluoromethylphenyl]-piperazin-1-yl}-1-(4-dichlorobenzyl)-2-oxoethyl]pyrrolidin-2-one trifluoroacetate (13). This compound was prepared using a procedure similar to that of **12e**, by replacing *R*-**14b** with *S*-**14b**. White foam, HPLC purity: 100% (220 and 254 nm); ^{1}H NMR (DMSO-d_6): 7.82 (d, J = 1.8 Hz, 1H), 7.50 (dd, J = 1.8, 8.8 Hz, 1H), 7.22–7.33 (m, 4H), 7.20 (d, J = 8.8 Hz, 1H), 5.10–5.18 (m, 1H), 4.33–4.41 (m, 1H), 3.40–3.72 (m, 2H), 3.20–3.32 (m, 4H), 2.75–3.07 (m, 6H), 2.60–2.72 (m, 1H), 2.00–2.27 (m, 1H), 1.76–2.00 (m, 2H), 1.39–1.58 (m, 2H), 1.24–1.36 (m, 1H), 0.86 (d, J = 6.1 Hz, 3H), 0.83 (d, J = 6.1 Hz, 3H); MS: 565 (MH^+).

1.2. 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.[13]

1.3. Pharmacokinetic characterization

The pharmacokinetic profile of **11**, **12a**, and **12e** was determined in male mice (N = 3/time points at a dose of 10 mg/kg for po or 5 mg/kg for iv). Compounds were dosed as a dihydrochloride salt (**11**) or mesylate salt (**12a** and **12e**) in 5% methylcellulose via the tail vain (iv) or in water with 5% v/v cremophor via oral gavage (po). Composite sampling was used to collect samples. Terminal blood samples were taken from treated mice at 9 time points ranging from pre-dose to 24 h (0, 0.25, 0.5, 1, 2, 4, 6, 8, and 24 h) post-dose. Brain samples were collected at 1 and 4 h post-iv dosing.

Plasma and brain sample analyses for the quantification of parent compounds were conducted using a HPLC equipped with a mass spectrometric detector (LC–MS/MS). For plasma samples, the compound was extracted via a protein precipitation assay by adding 130 mL of acetonitrile (CAN) and 30 mL of internal standard in 50 μL of mouse plasma (EDTA). Standards and QCs were prepared by drying 50 mL of spiked aqueous solutions and reconstituting with 50 mL of blank mouse plasma. For brain samples, the whole brain was homogenized in 2.0 mL of ACN/H_2O/formic acid (v/v: 60:40:0.1) containing 50 mL of internal standard, and the supernatant was collected and injected into an LC–MS/MS system for analysis. Brain standards and QCs were prepared by adding 1.0 mL of spiked aqueous solutions (prepared in 60:40 ACN/H_2O), 1.0 mL of ACN/H_2O/formic acid (v/v: 60:40:0.1), and 50 mL of internal standard into a whole blank mouse brain. Standards and QCs for both plasma and brain were processed and analyzed at the same time and in exactly the same way as the analytical samples. The compound was measured using a specific and sensitive HPLC/MS assay that offered linear ranges of 1–1000 ng/mL for iv/po plasma sample analysis, and 1–500 ng/mL for iv brain sample analysis. The Lower Limit of Quantitation (LLOQ) was 1 ng/mL for the study. Quantification for both plasma and brain samples was performed by fitting peak area ratios to a weighted ($1/x$) linear calibration curve.

Descriptive pharmacokinetics was derived and evaluated based on the mean plasma concentrations ($N = 3$/time point). A non-compartmental model in ActivityBase with linear trapezoidal rule was used to perform all pharmacokinetic analyses pertained to this manuscript.

1.4. Efficacy study

C57BL/6 J male mice, obtained from Jackson Laboratories (Bar Harbor, Maine), were used for the cachexia studies. Mice were housed individually and maintained on powdered Purina 5015 chow (13% fat) for at least seven days prior to the start of the study. All studies were conducted according to the NIH Guide for the Care and Use of Laboratory Animal and approved by the Animal Care and Use Committee of the Oregon Health Sciences.

As previously described,[3a] on day 0, mice were inoculated subcutaneously into the upper flank with 1×10^6 cells from a subcloned Lewis lung carcinoma (LLC) cell line. All experimental animals were found to have a palpable tumor within 5 days of the start of the experiment. At the time of sacrifice, tumors were dissected away from surrounding tissue and weighed. There was no statistical difference in tumor size between treatment groups and gross examination of organs did not reveal the presence of metastasis.

On day 11 after tumor inoculation, mice were divided into 3 groups and treated twice daily with vehicle, 3, or 9 mg/kg **12e** (ip). Food intake and body weight were measured daily. Following 4 days of treatment, animals were sacrificed, tumors were removed, and body composition was determined by dual-energy X-ray absorbtometry (DEXA, PIXImus mouse densitometer, Lunar corp.). A baseline measure was made on the day of inoculation (day 0) also. Animals were anesthetized prior to the first scan, and asphyxiated with CO_2 prior to the tumor dissection and the final scan.

Food intake was averaged over the treatment days. Percent change in body weight, lean mass, and fat mass was computed from the initial day of the experiment to the final day. Differences among groups for these variables were analyzed by one-way ANOVAs with post hoc analysis. Data sets were analyzed for statistical significance using SigmaStat (SPSS, Inc.).

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