European Journal of Medicinal Chemistry 46 (2011) 5310–5316

Contents lists available at SciVerse ScienceDirect

European Journal of Medicinal Chemistry

journal homepage: http://www.elsevier.com/locate/ejmech

Original article

Asymmetric synthesis and biological evaluation of *N*-cyclohexyl-4-[1-(2,4-dichlorophenyl)-1-(*p*-tolyl)methyl]piperazine-1-carboxamide as hCB1 receptor antagonists

Linghuan Gao[a,b,1], Min Li[a,1], Tao Meng[a,*], Hongli Peng[a,*], Xin Xie[a], Yongliang Zhang[a], Yu Jin[a], Xin Wang[a], Libo Zou[b], Jingkang Shen[a,*]

[a] State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, PR China
[b] Department of Pharmacology, School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shengyang 110016, PR China

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form
19 July 2011
Accepted 20 August 2011
Available online 1 September 2011

Keywords:
Anti-obesity
CB1 receptor
Antagonist
Asymmetric synthesis
Enantiomer

ABSTRACT

We recently discovered and reported a novel series of benzhydrylpiperazine derivatives bearing an asymmetric carbon atom that are potent and selective hCB1 inverse agonists. In the present study, we used Davis-Ellmann-type sulfonamide chemistry to asymmetrically synthesize two enantiomers of the most potent racemic *N*-cyclohexyl-4-[1-(2,4-dichlorophenyl)-1-(*p*-tolyl)methyl]piperazine-1-carboxamide [14]. Enantiomer separation and configuration assignment were carried out. Our results indicate that the ***R***-configuration is the more active enantiomer, displaying enhanced antagonistic activity for hCB1 receptor, better oral bioavailability, and greater efficacy in the reduction of body weight in diet-induced obese mice.

1. Introduction

Endocannabinoids increase food intake through their interaction with the cannabinoid receptor 1 (CB1) expressed in the brain. Pharmacological blockade of CB1 has shown promising results in the treatment of obesity, type 2 diabetes, and metabolic syndromes [1–3]. Rimonabant (SR141716, Acomplia™, Fig. 1), a potent and selective CB1 receptor antagonist/inverse agonist, was launched by Sanofi-Aventis in Europe in 2006 for the treatment of obesity and associated risk factors [4]. However, it was associated with psychiatric side effects such as depression, anxiety and stress disorders [5], which led to its withdrawal from the European market in 2008, and also the withdrawal of several other CB1 inverse agonists including taranabant and otenabant from phase III clinical trials [6]. Although the exact role of the endocannabinoid system in the control of mood and anxiety-like behaviors is not clear, CB1 modulation in the brain has been suggested to be relevant in these behaviors [7]. On the other hand, CB1 is also found in the adipose tissue, skeletal muscle, peripheral nerves, and digestive system (gastrointestinal tract, pancreas and liver) [8,9], where its activation contributes to obesity-related metabolic and hormonal abnormalities [9]. It has been reported that liver-specific CB1 knockout ($LCB1^{-/-}$) mice are resistant to diet-induced obesity [10], indicating that activation of CB1 in peripheral liver tissue contributes to the diet-induced steatosis and associated metabolic changes. Chronic treatment of obese mice or rats with a CB1 antagonist induces sustained weight loss and protects rodents from this detrimental metabolic phenotype, even though it causes only a transient reduction in food intake [11]. Clinical trials also showed that rimonabant can significantly improve glycemic index and dyslipidemia in type 2 diabetic patients, and the profile of several other metabolic risk factors including the reduction of visceral and hepatic fat in overweight and obese patients [12,13]. These findings indicate that CB1 can directly affect peripheral energy metabolism by mechanisms unrelated to their effect on appetite, suggesting that selective targeting of peripheral CB1 may result in an improved metabolic profile in obesity without the untoward behavioral effects observed following treatment with brain-acting CB1 antagonists.

* Corresponding authors. Tel.: +86 21 50806600/5407; fax: +86 21 50807088.
E-mail addresses: tmeng@mail.shcnc.ac.cn (T. Meng), penghl@mail.shcnc.ac.cn (H. Peng), jkshen@mail.shcnc.ac.cn (J. Shen).
[1] These authors contributed equally to this work.

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doi:10.1016/j.ejmech.2011.08.030

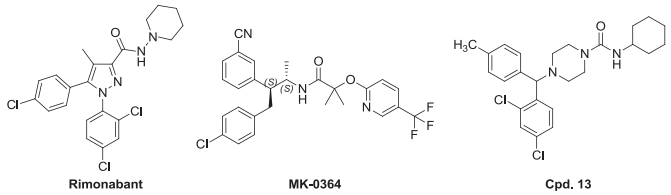


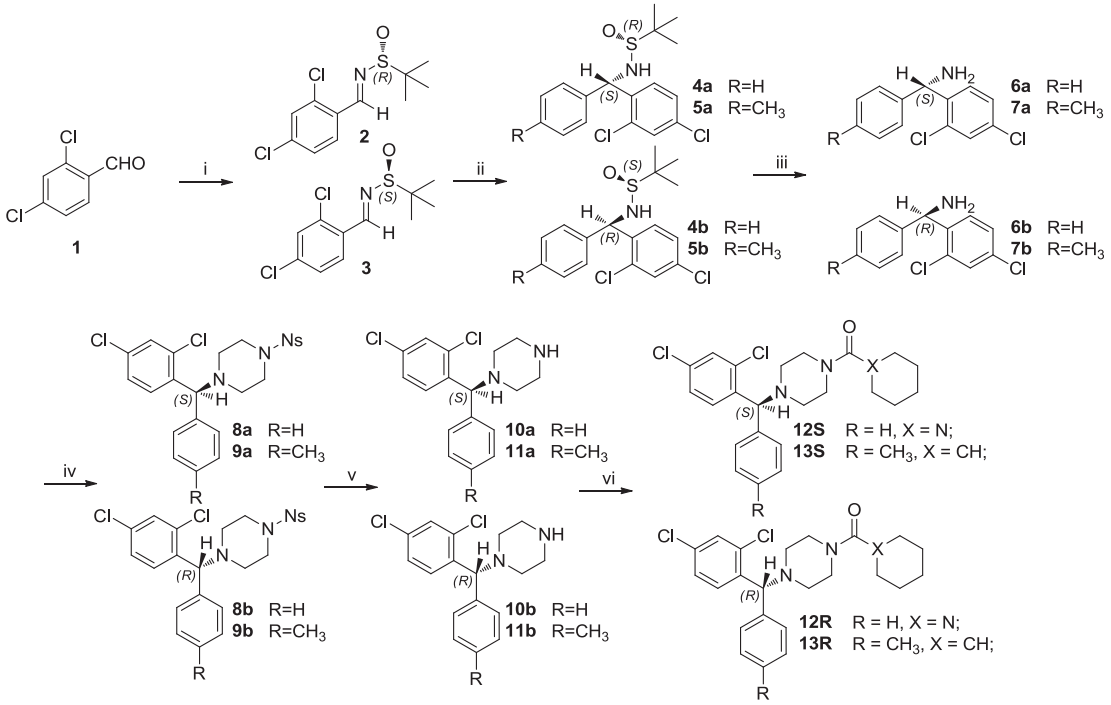
Fig. 1. CB1 receptor inverse agonists.

Recently, we have developed a novel series of benzhydrylpiperazine derivatives, *via* privileged structure-based approach, leading to the identification of compound **13** (Fig. 1) as a highly potent and selective hCB1 inverse agonist (K_{iCB1} = 0.15 nM; EC_{50} = 0.87 nM) [14]. Similar analogs were also reported by Song et al. [15]. We found that, although compound **13** exhibited a lower brain exposure compared to rimonabant (the brain/plasma ratios are 1.0 and 4.4 for compound **13** and rimonabant, respectively), it displayed comparable body weight-loss efficacy in diet-induced obese (DIO) rats [14] (rimonabant data not shown). This strongly supported the concept mentioned above that efficacious CB1 antagonists for the treatment of obesity are practicable without central nervous system (CNS) liability. All the benzhydrylpiperazine analogs examined in our previous structure-activity relationships (SAR) studies featured a stereocenter. It is well known for chiral drugs that their enantiomers can differ in pharmacodynamic properties and/or biological activities, and in most cases only one of the enantiomers, the eutomer, is responsible for therapeutic effect [16]. Since it has been reported that there was a 2- to 10-fold difference in affinity to hCB1 between the *anti*- and *syn*-diastereomers of CB1R inverse agonist, MK-0364 (Fig. 1) [17], it was necessary to undertake a further investigation of the stereochemical consequences for pharmacokinetic profiles and bioactivities of compound **13**. Herein, we wish to report our efforts on the asymmetric synthesis of compounds **13S** and **13R** *via* Davis-Ellmann-type sulfonamide chemistry [18], the pharmacokinetic profiles and the *in vitro* and *in vivo* activities of the enantiomers.

2. Results and discussion

2.1. Chemistry

Chiral resolution is commonly used for the separation of racemic compounds into their enantiomers. Opalka et al [19] has described a separation of a (±)-4-chlorobenzhydrylamine by co-recrystallization with L(+)-tartaric acid. Initially, we tried to resolve a racemic (2,4-dichlorophenyl)(*p*-tolyl)methanamine into its enantiomers (**7a** and **7b**) by fractional crystallization of its L-(+)-tartaric acid or D-(−)-tartaric acid salts using a similar procedure reported by Opalka [19]. However, after six times of co-recrystallization from the water-ethanol or water-methanol mixture, the enantiomeric (2,4-dichlorophenyl)(*p*-tolyl)methanamine is obtained in only up to 20% enantiomer excess determined by chiral HPLC. This approach is inapplicable for the preparation of the intermediates of enantiomers **7a** and **7b**. Therefore, asymmetric synthesis of the major diastereomers of **5a** and **5b** (Scheme 1) was investigated. Davis-Ellmann-type sulfonamide chemistry, as a practical asymmetric process for the preparation of chiral non-racemic amines, has been applied to the asymmetric synthesis of levocetirizine [20–23]. Sulfinimines have also shown to be efficient chiral auxiliaries for the preparation of chiral di-substituted benzhydrylamines [22,23]. As depicted in Scheme 1, Ti(O-*i*Pr)$_4$-mediated synthesis of (R)/(S)-*N*-*tert*-butanesulfinyl aldimines(**2**, **3**) from (*R*)-t-butyl sulfonamide [(*R*)-TBS] or (*S*)-t-butyl sulfonamide [(*S*)-TBS] and 2,4-dichlorobenzaldehyde proceeded in high yields. With the addition of arylmagnesium bromides to R)/(S)-*N*-*tert*-



Scheme 1. Asymmetric synthesis route of the enantiomers of compounds **12** and **13**. Reagents and conditions: (i) for compound **2**: (*R*)-*t*-butyl sulfonamide, Ti(O-*i*Pr)$_4$, THF, 93%; for compound **3**: (*S*)-*t*-butyl sulfonamide, Ti(O-*i*Pr)$_4$, THF, 92%; (ii) ArMgBr, THF/toluene, −20 °C-0 °C, 5 h, 74%∼81%; (iii) saturated HCl in MeOH, 2 h, 96%∼98%; (iv) *N*,*N*-bis(2-chloroethyl)-2-nitrobenzenesulfonamide, DIPEA, 130 °C, 4 h, 66%∼74%; (v) PhSH, K_2CO_3, acetonitrile, 30 °C, 6 h, 65%∼75%; (vi) for compound **12S** and **12R**: 1-aminopiperidine, 1,1′-carbonyldiimidazole (CDI), THF, rt, 82% and 78%, respectively; for compound **13S**, **13R**: cyclohexyl isocyanate, CH_2Cl_2, rt, 89% and 86%, respectively.

butanesulfinyl aldimines(**2**, **3**) at −20 °C, followed by aging at room temperature in toluene, the diastereomers of intermediates **5a** and **5b** were obtained with approximate 85:15 diastereoselectivity observed by HPLC analysis, which was similar to the results reported by Pflum et al. [22]. Since compounds **5a** and **5b** are oil-like products, it is difficult to determine the absolute configuration of the newly formed stereocenters. Thus, the corresponding analogs without methyl group, the diastereomers of **4a** and **4b**, were synthesized and crystallized from petroleum ether to provide diastereopure material in over 70% yield. Single crystal X-ray analysis shown in Fig. 2 confirmed that the absolute configuration of the newly formed stereocenter in compound **4a** was (*S*). Thereafter, the absolute stereochemistry of analogs with methyl group (**7a/b, 11a/b, 13a/b**) can be established by comparing their optical rotatory dispersion with that of the corresponding *S*(−)/*R*(+)-analogs without methyl group (**6a/b, 10a/b, 12a/b**) [24].

After removal of the chiral auxiliary by a mild hydrolysis condition (saturated HCl in methanol), the products with L(+)-tartaric acid or D(−)-tartaric acid were further diastereomerically crystallized to afford intermediates *S*(−)-amine **6a** and *R*(+)-amine **6b**, as well as **7a** and **7b** with higher enantiomerical purity determined by optical rotatory mearurement. According to the asymmetric synthesis route of levocetirizine, the ring closure reaction product of the (−)4-chlorobenzhydrylamine and *N,N*-bis(2-chloroethyl)-4-methylbenzenesulfonamide was subjected to a standard *N*-tosyl deprotection procedure with 40% HBr in acetic acid solution to afford piperazine product [18,21]. However, we found that the *N*-tosyl protecting group could not be removed from *N*-tosyl protected piperizines **14** and **15** with HBr/HOAc (Scheme 2). Thus, *N*-nosyl was used as the amine protecting group to replace the tosyl group. The bisalkylation of (*S*)-amine **6a**, **7a** or (*R*)-amine **6b**, **7b** with *N,N*-bis(2-chloroethyl)-2-nitrobenzenesulfonamide, followed by mild deprotection condition, afforded **10a**, **11a** or **10b**, **11b**. The piperazine products were reacted with cyclohexylisocyanate and 1-aminopiperidine, respectively, to give **12S/12R** in over 96% ee, the desired products **13S/13R** in over 95% ee

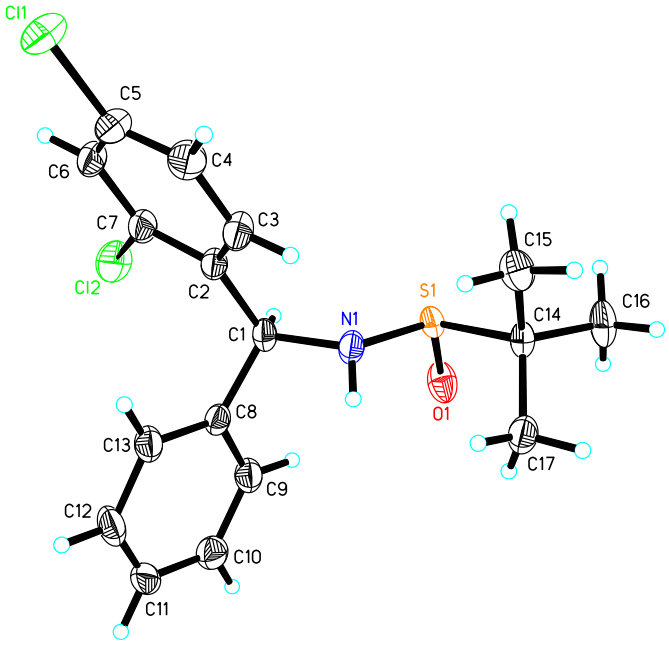
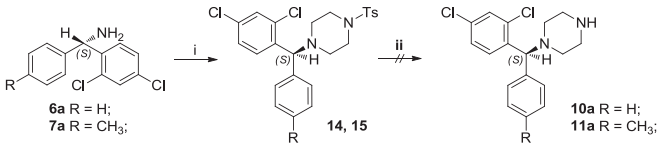


Fig. 2. X-Ray single crystal structure of (*R,S*)-sulfinamide-4a (major disastereomer from PhMgBr addition process).



Scheme 2. Initial proposed synthesis of compound **10a** and **11a**. Reagents and conditions: (i) *N,N*-bis(2-chloroethyl)-4-methylbenzenesulfonamide, DIPEA, 170 °C, 3 h; (ii) 40% HBr in HOAc (various phenols used in deprotection: 10% phenol/20 °C, or 10% 4-hydroxybenzoic acid/20 °C, or 10% phenol/80 °C (HPLC yield: trace ~ 5%).

(Scheme 1), indicating that there was no epimerization of the stereocenter during the acidic hydrolysis [24].

2.2. In vitro assay

The target compounds were evaluated *in vitro* on the hCB1 and hCB2 receptors, stably expressed in Chinese Hamster Ovary (CHO) cells, utilizing radioligand binding studies. hCB1 receptor antagonism was measured using a CP-55940 (an agonist of the CBRs) induced Ca^{2+} increase functional assay in CHO cells co-expressing hCB1 receptor and Gα15/16.

A comparison between the two enantiomers **13R** and **13S** and the corresponding racemate **13** showed that the two single enantiomers **13R** and **13S** differed in their binding affinities for hCB1 by 5-fold, whereas the *R*-enantiomer was the more active form (**13R**, $K_{iCB1} = 0.2$ nM; **13S**, $K_{iCB1} = 1.1$ nM) and had a potency comparable to that of the racemic compound **13** ($K_{iCB1} = 0.15$ nM, Table 1). Similar results were observed in the antagonistic activities by a functional Ca^{2+} assay, in which again, the enantiomer **13R** is the more active form and displayed 3-fold improvement in the inhibition of CP-55940-induced Ca^{2+} increase. Meanwhile, the enantiomer **13S** exhibited a significant 8-fold decrease in Ca^{2+} inhibitory potency, compared to the racemic compound **13** (Table 1).

2.3. Pharmacokinetic and in vivo studies

The pharmacokinetic profiles of the racemic compounds **13**, and its enantiomers **13R** and **13S** after oral administration were evaluated in Sprague–Dawley rats. As shown in Table 2, it was

Table 1
Assay results of enantiomers of the racemate **13**.[a]

Cpd.	Absolute configuration (optical rotation)	K_i hCB1 (nM)	K_i hCB2 (nM)	IC_{50} hCB1 Ca^{2+} (nM)
13 [14]	/	0.15 ± 0.04	329 ± 71	2.7 ± 0.5
13S	*S* (+4.3°)	1.1 ± 0.1	242.4 ± 119.0	21.5 ± 8.3
13R	*R* (−4.3°)	0.2 ± 0.03	226.8 ± 45.9	0.9 ± 0.4

[a] K_i and IC_{50} (mean ± SEM) ($n = 3$ independent experiments) were calculated from dose–response curves. hCB1: human CB1 receptor. hCB2: human CB2 receptor.

Table 2
Pharmacokinetic studies of the racemic compound **13**, and its enantiomers **13S** and **13R** in Sprague–Dawley rats after 10 mg/kg p.o. administration.[a]

Cpd.	**13**	**13S**	**13R**
t_{max} (h)	2.4 ± 0.2	1.3 ± 0.3	1.2 ± 0.3
C_{max} (ng/mL)	179.5 ± 31.3	128.5 ± 10.5	115.7 ± 19.3
$t_{1/2}$ (h)	2.3 ± 0.1	3.4 ± 1.0	3.8 ± 0.6
$AUC_{0-\infty}$ (ng h/mL)	883 ± 112	695 ± 34	655 ± 68
F (%)	8%	6%	15%
B/P @ 9 h postdose	0.91 ± 0.07	1.42 ± 0.17	0.88 ± 0.06

[a] Values represent the mean ± SEM for at least three experiments. C_{max}: maximum plasma concentration of drug; t_{max}: time taken to reach C_{max}; $t_{1/2}$: half-life; $AUC_{0-\infty}$: area under the curve; *F*: oral bioavailability.

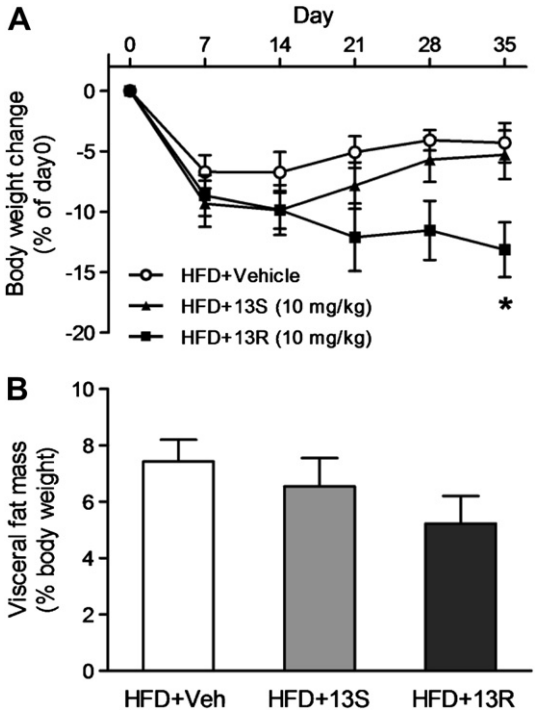


Fig. 3. Chronic effects after oral administration of the enantiomers 13S and 13R in DIO mice. A: weekly body weight; B: visceral fat mass at end of study.*$P < 0.05$ *vs*. vehicle, two-way ANOVA followed by Bonferroni posttests. Data represent mean ± SE ($n = 8$–10 mice/group).

found that, in addition to displaying similar *in vitro* biological activities, the enantiomer **13R** ($t_{1/2} = 3.8$ h; brain/plasma = 0.88) and its racemic compounds **13** ($t_{1/2} = 2.3$ h; brain/plasma = 0.91) also had similar pharmacokinetic profiles such as halflife ($t_{1/2}$) and brain exposure, but the enantiomer **13R** was found to have almost twofold improvement in oral bioavailability (*F*% = 15% *vs*. *F*% = 8% for **13R** and **13**, respectively). The enantiomer **13S** displayed poor pharmacokinetic profiles as decreased oral bioavailability (*F* % = 6%) and increased brain exposure (brain/plasma = 1.42). Based on the above results, the enantiomer **13R** would account for the majority of the inhibitory potency of its racemate **13**, and is expected to have greater *in vivo* efficacy than the enantiomer **13S**. This was indeed the case, as seen in Fig. 3 where the effects of **13R** and **13S** on body weight were evaluated in DIO mice. Long-term applications of **13R** (10 mg/kg, p.o.) once daily for 35 days led to a steady reduction of body weight and caused a significance body weight loss at the end of the 35-day study compared to vehicle treatment (Fig. 3A), while 10 mg/kg of **13S** group displayed no effect on body weight reduction. The **13R** group also showed a reduction of the visceral fat mass of DIO mice (Fig. 3B), although it did not achieve statistical significance. Overall food intake did not differ between **13R** and **13S** groups in this long-term experiment (data not shown).

3. Conclusions

In an important extension of previous work, we have demonstrated a practical process for the assymetric synthesis of benzhydrylpiperazines **13S** and **13R** as potential CB1 antagonists. The pharmacokinetic profiles and the *in vitro* and *in vivo* activities of the enantiomers **13S** and **13R** had been evaluated, and **13R** was recognized as the more potent form. It also displayed enhanced antagonistic activity for hCB1, better oral bioavailability, and greater efficacy under *in vivo*. The relationship between activity and stereochemistry provided us with useful information in our search for peripherally acting CB1 antagonists, which will be reported in due course.

4. Experiment procedure

4.1. Chemistry

All non-aqueous reactions were performed in flame-dried glassware under an atmosphere of dry Ar, unless otherwise specified. Tetrahydrofuran (THF) was distilled from sodium-benzophenone ketyl under an atmosphere of Ar immediately prior to use; Dichloromethane (DCM) was freshly distilled from calcium hydride under Ar. All other solvents were reagent grade. Petroleum ether refers to a mixture of alkanes with the boiling range 60–90 °C ^{1}H and ^{13}C NMR spectra were recorded on Varian Mercury-300 and Varian Mercury-400 spectrometers. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard. The following abbreviations were used to explain the multiplicities: s = singlet, d = doublet, t = triplet, q = quartet, dd = doublet of doublets, m = multiplet, br = broadened. Infrared spectra were recorded on a Nicolet 750FT-IR. Optical rotations were measured on a Perkin-Elmer model 341 polarimeter. The LC-MS were carried out on Thermo Finnigan LCQDECAXP and low-resolution mass spectrometry was performed on Finnigan MAT 95 and Finnigan LCQ Deca spectrometers, high resolution mass spectra were measured on Finnigan MAT 95 and MicroMass Q-Tof ultima™ mass spectrometers. Optical rotations were determined on a Perkin–Elmer 341 polarimeter.

4.1.1. (*R*)-*N*-(2,4-dichlorophenyl)methylene-2-methylpropane-2-sulfinamide (**2**)

A solution of Ti(O-*i*Pr)$_4$ (247 mL, 0.83 mol) and 2,4-dichlorobenzaldehyde (72.2 g, 0.41 mol) in 500 mL of THF was prepared under a N_2 atmosphere. Then, (*R*)-*t*-butyl sulfonamide (50.0 g, 0.41 mol) was added and the reaction mixture was stirred at 40 °C for 6 h (conversion was followed by TLC). And the mixture cooled immediately upon completion. Once at room temperature, the mixture was poured into 500 mL of brine while rapidly stirring. The resulting suspension was filtered through a plug of Celite, and the filter cake was washed with EtOAc. The filtrate was transferred to a separatory funnel where the organic layer was washed with brine. The brine layer was extracted once with a small volume of EtOAc, and the combined organic portions were dried over Na_2SO_4, filtered, and concentrated. Compound **2** was obtained as white solid form the residue by recrystallization from petroleum ether and ethyl acetate (106.3 g, 93%). $[\alpha]_D^{20} = -178.7$ (c 1.0, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.97(s, 1H), 8.01(d, $J = 8.5$ Hz, 1H), 7.48(d, $J = 2.1$ Hz, 1H), 7.34 (ddd, $J = 8.5$, 2.0, 0.7 Hz, 1H), 1.28 (s, 9H). LRMS(EI, 70 eV): *m*/*z* 277 [M^+].

4.1.2. (*S*)-*N*-(2,4-dichlorophenyl)methylene-2-methylpropane-2-sulfinamide (**3**)

Compound **3** was prepared from 2,4-dichlorobenzaldehyde and (*S*)-*t*-butyl sulfonamide in 92% yield as white solid by the same procedure as described for intermediate **2**. $[\alpha]_D^{20} = +177.6$ (c 1.0, $CHCl_3$), other physical and chemical data were identical with those described above for the opposite enantiomer **2**.

4.1.3. (*R*)-*N*-[(*S*)-(2,4-dichlorophenyl)(phenyl)methyl]-2-methylpropane-2-sulfinamide (**4a**)

To a solution of the sulfinimine **2** (30.0 g, 0.11 mol) in 200 mL of toluene was added 50 mL of 2.8 M phenylmagnesium bromide tetrahydrofuran solution at −20 °C. The mixture was stirred at

0 °C for 3 h and then was allowed to warm to room temperature and stirred overnight. The reaction mixture was quenched with 10 mL of saturated ammonium chloride aqueous solution and the aqueous layer was extracted with ethyl acetate (2 × 250 mL). The combined organic layers were washed with water and brine, dried over Na_2SO_4, and concentrated to provide crude product, which was recrystallized from petroleum ether to give the pure **4a** as white solid (28.3 g, 74%). mp 104–106 °C $[\alpha]_D^{20} = -47.0$ (c 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ 7.57 (d, J = 8.5 Hz, 1H), 7.38 (d, J = 2.2 Hz, 1H), 7.28–7.34 (m, 6H), 6.07 (d, J = 2.8 Hz, 1H), 3.67(d, J = 2.8 Hz, 1H, NH), 1.26 (s, 9H). LRMS(EI, 70 eV): m/z 355 [M^+], 235 (100%).

4.1.4. (R)-N-[(S)-(2,4-dichlorophenyl)(p-tolyl)methyl]-2-methylpropane-2-sulfinamide (**5a**)

Compound **5a** was prepared from sulfinimine **2** and *p*-tolylmagnesium bromide in 81% yield as yellow oil by the same procedure as described for **4a**. $[\alpha]_D^{20} = -44.9$ (c 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ 7.58 (d, J = 8.4 Hz, 1H), 7.36 (d, J = 2.2 Hz, 1H), 7.29 (dd, J = 8.4 Hz, 2.1 Hz, 1H), 7.11–7.23 (m, 4H), 6.03 (d, J = 2.8 Hz, 1H), 3.64 (d, J = 2.4 Hz, 1H), 2.31 (s, 3H), 1.25 (s, 9H). LRMS(EI, 70 eV): m/z 369[M^+], 249 (100%).

4.1.5. (S)-N-[(R)-(2,4-dichlorophenyl)(phenyl)methyl]-2-methylpropane-2-sulfinamide (**4b**)

Compound **4b** was prepared from sulfinimine **3** and phenylmagnesium bromide in 76% yield as white solid by the same procedure as described for **4a**. $[\alpha]_D^{20} = +47.3$ (c 1.0, $CHCl_3$), other physical and chemical data were identical with those of **4a**.

4.1.6. (S)-N-[(R)-(2,4-dichlorophenyl)(p-tolyl)methyl]-2-methylpropane-2-sulfinamide (**5b**)

Compound **5b** was prepared from sulfinimine **3** and *p*-tolylmagnesium bromide in 80% yield as yellow oil by the same procedure as described for **4a**. $[\alpha]_D^{20} = +45.5$ (c 1.0, $CHCl_3$), other physical and chemical data were identical with those of **5a**.

4.1.7. (S)-(2,4-dichlorophenyl)(phenyl)methanamine (**6a**)

Compound **4a** (20.0 g, 54 mmol) was treated with a saturated HCl in methanol (200 mL) at room temperature for 2 h. The mixture was concentrated to dryness and precipitated with diethyl ether. The precipitate was collected by filtration and washed with diethyl ether to give the amine hydrochloride of sufficient purity such that no other purification was required. The amine hydrochloride was converted to the free amine by treatment with 1 N $NaOH_{(aq.)}$ (100 mL), and was extracted with CH_2Cl_2 (2 × 150 mL), the combined organic layer was washed with H_2O and brine successively, dried over Na_2SO_4 and evaporated under vacuum to get the product as pale yellow oil (13.8 g, 98%). $[\alpha]_D^{20} = -7.6$ (c 1.0, EtOH). ^1H NMR (400 MHz, $CDCl_3$) δ 7.52 (d, J = 8.4 Hz, 1H), 7.23–7.38 (m, 7H), 5.60 (s, 1H). LRMS(EI, 70 eV): m/z 251[M^+].

4.1.8. (S)-(2,4-dichlorophenyl)(p-tolyl)methanamine (**7a**)

Compound **7a** was prepared from **5a** in 97% yield by the same procedure as described for **11a**. $[\alpha]_D^{20} = -10.2$ (c 1.0, EtOH). ^1H NMR (300 MHz, $CDCl_3$) δ 7.52 (d, J = 8.4 Hz, 1H), 7.33 (d, J = 2.2 Hz, 1H), 7.21–7.25 (m, 3H), 7.10–7.13 (br d, J = 7.9 Hz, 2H), 5.55 (s, 1H), 2.32 (s, 3H). LRMS(EI, 70 eV): m/z 265[M^+].

4.1.9. (R)-(2,4-dichlorophenyl)(phenyl)methanamine (**6b**)

Compound **6b** was prepared from **4b** in 98% yield by the same procedure as described for **11a**. $[\alpha]_D^{20} = +7.6°$ (c 1.0, EtOH), other physical and chemical data were identical with those described above for the opposite enantiomer.

4.1.10. (R)-(2,4-dichlorophenyl)(p-tolyl)methanamine (**7b**)

Compound **7b** was prepared from **5b** in 96% yield by the same procedure as described for **11a**. $[\alpha]_D^{20} = +10.1$ (c 1.0, EtOH), other physical and chemical data were identical with those described above for the opposite enantiomer.

4.1.11. 1-[(S)-(2,4-dichlorophenyl)(phenyl)methyl]-4-[(2-nitrophenyl)sulfonyl]piperazine (**8a**)

To the free base of (*S*)-(2,4-dichlorophenyl)(phenyl)methanamine (**6a**, 44 g, 0.18 mol) was added *N*,*N*-diisopropylethylamine (33.5 mL, 0.19 mol), to this mixture was added *N*,*N*-bis(2-chloroethyl)-2-nitrobenzenesulfonamide (57 g, 0.18 mol, prepared from bis(2-chloroethyl)amine hydrochloride and 2-nitrobenzenesulfonyl chloride in 96% yield), carefully in portions to ensure mixing. After the addition of the bis-alkylating agent was complete, the flask was then equipped with a reflux condenser and drying tube, and the mixture was stirred at 130 °C for 4 h. The reaction mixture turned from yellow to brown, and was allowed to cool to room temperature. The reaction mixture was redissolved in 300 mL of DCM, the solution was washed with H_2O (3 × 100 mL), then with brine (100 mL), dried over Na_2SO_4 and evaporated under vacuum to afford the crude product **8a** (65.3 g, 74%), which was used in the next step without purification, LC-MS (major peak): 506.1[M+1].

4.1.12. 1-[(R)-(2,4-dichlorophenyl)(phenyl)methyl]-4-[(2-nitrophenyl)sulfonyl]piperazine (**8b**)

Compound **8b** was prepared from **6b** in 70% yield by the same procedure as described for **8a**. LC-MS (major peak): 506.1[M+1].

4.1.13. 1-[(S)-(2,4-dichlorophenyl)(p-tolyl)methyl]-4-[(2-nitrophenyl)sulfonyl]piperazine (**9a**)

Compound **9a** was prepared from **7a** in 66% yield by the same procedure as described for **8a**. LC-MS (major peak): 520.1[M+1].

4.1.14. 1-[(R)-(2,4-dichlorophenyl)(p-tolyl)methyl]-4-[(2-nitrophenyl)sulfonyl]piperazine (**9b**)

Compound **9b** was prepared from **7b** in 71% yield by the same procedure as described for **8a**. LC-MS (major peak): 520.1[M+1].

4.1.15. 1-[(S)-(2,4-dichlorophenyl)(phenyl)methyl]piperazine (**10a**)

To a solution of the crude 1-[(*S*)-(2,4-dichlorophenyl)(phenyl)methyl]-4-[(2-nitrophenyl)sulfonyl] piperazine (**6a**, 60.0 g, approx. 0.12 mol) in acetonitrile (150 mL) at 0 °C was added thiophenol (14.6 mL, 0.14 mol) and potassium carbonate (50 g, 0.36 mol), the mixture was stirred at 50 °C for 2 h, then the solvent was removed in vacuum. The residue was dissolved in 300 mL ethyl acetate and 150 mL water, the organic layer was washed 100 mL water, followed by 1 N HCl (200 mL), then the aqueous phase was washed ethyl acetate (3 × 80 mL), and the combined organic phase was discarded, the aqueous phase was basified with 3 N $NaOH_{aq.}$ (80 mL) to pH = 10, then the aqueous solution was extracted with CH_2Cl_2 (3 × 100 mL). The combined organic phase was washed successively with water, brine, dried over Na_2SO_4 and evaporated under vacuum to provide the title compound **10a** (27.3 g, 72%) as yellow oil. $[\alpha]_D^{20} = -4.6$ (c 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ 7.74 (d, J = 8.4 Hz, 1H), 7.37–7.40 (m, 2H), 7.18–7.31 (m, 5H), 4.80 (s, 1H), 3.02–3.08 (m, 3H), 2.26–2.61 (m, 5H). LRMS(EI, 70 eV): m/z 320 [M^+].

4.1.16. 1-[(S)-(2,4-dichlorophenyl)(p-tolyl)methyl]piperazine (**11a**)

Compound **11a** was prepared from **9a** in 65% yield by the same procedure as described for **10a**. $[\alpha]_D^{20} = -5.5$ (c 1.0, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$) δ 7.77 (d, J = 8.4 Hz, 1H), 7.22–7.29 (m, 4H), 7.07–7.09 (m, 2H), 4.71 (s, 1H), 2.28–2.95 (m, 4H), 2.39 (br s, 4H), 2.28 (s, 3H). LRMS(EI, 70 eV): m/z 334 [M^+].

*4.1.17. 1-[(R)-(2,4-dichlorophenyl)(phenyl)methyl]piperazine (**10b**)*

Compound **10b** was prepared from **8b** in 73% yield by the same procedure as described for **10a**. $[\alpha]_D^{20} = +4.6$ (c 1.0, $CHCl_3$), other physical and chemical data were identical with those described above for the opposite enantiomer **10a**.

*4.1.18. 1-[(R)-(2,4-dichlorophenyl)(p-tolyl)methyl]piperazine (**11b**)*

Compound **11b** was prepared from **9b** in 75% yield by the same procedure as described for **10a**. $[\alpha]_D^{20} = +5.4$ (c 1.0, $CHCl_3$), other physical and chemical data were identical with those described above for the opposite enantiomer **11a**.

*4.1.19. 4-[(S)-(2,4-dichlorophenyl)(phenyl)methyl]-N-piperidin-1-ylpiperazine-1-carbox-amide (**12S**)*

Compound **12S** was prepared from *N,N'*-carbonyl diimidazole (CDI), 1-aminopiperidine and **10a** in 82% yield as white solid by the same procedure as described previously [14]. $[\alpha]_D^{20} = +2.8$ (c 1.0, EtOH), >99% ee (analyzed on an 'Chiralpak AD-H' column, flow rate 1.0 mL/min, temperature 30 °C, $\lambda = 254$ nm, hexane/*i*-PrOH, 97:3 with 0.1% diethylamine, $t_{R\ (major)} = 29.2$ min, minor one is not observed). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.77 (d, $J = 8.4$ Hz, 1H), 7.38–7.42 (m, 2H), 7.19–7.31 (m, 5H), 4.97 (s, 1H, NH), 4.74 (s, 1H), 3.44–3.47 (m, 4H), 2.65 (br s, 4H), 2.35–2.38 (m, 4H), 1.58–1.66 (m, 4H), 1.36 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 158.28, 140.20, 138.54, 134.34, 132.93, 129.73, 129.43, 128.56 × 2, 128.30 × 2, 127.52, 127.47, 69.93, 57.62 (2 × CH_2), 51.66 (2 × CH_2), 44.71 (2 × CH_2), 25.70 (2 × CH_2), 23.21 (CH_2). LRMS(EI, 70 eV): *m/z* 446 [M^+].

*4.1.20. N-cyclohexyl-4-[(S)-(2,4-dichlorophenyl)(p-tolyl)methyl] piperazine-1-carboxamide (**13S**)*

Compound **13S** was prepared from **11a** and cyclohexyl isocyanate in 89% yield as white solid by the same procedure as described previously [14]. $[\alpha]_D^{20} = +4.3$ (c 1.0, EtOH), 98% ee (analyzed on an 'Chiralpak AD-H' column, flow rate 1.0 mL/min, temperature 30 °C, $\lambda = 254$ nm, hexane/*i*-PrOH, 97:3 with 0.1% diethylamine, $t_{R\ (major)} = 35.0$ min, $t_{R\ (minor)} = 39.3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.76 (d, $J = 8.4$ Hz, 1H), 7.23–7.29 (m, 4H), 7.07–7.09 (m, 2H), 4.70 (s, 1H), 4.21 (d, $J = 7.7$ Hz, 1H, NH), 3.29–3.32 (m, 4H), 2.35–2.37 (m, 4H), 2.29 (s, 3H), 1.91–1.95 (m, 2H), 1.66–1.71 (m, 2H), 1.59–1.62 (m, 2H), 1.31–1.38 (m, 2H), 1.03–1.14 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 157.04, 138.75, 137.22, 137.07, 134.27, 132.83, 129.57, 129.43, 129.27 × 2, 128.22 × 2, 127.50, 69.58, 51.38 (2 × CH_2), 49.31, 43.80 (2 × CH_2), 33.93 (2 × CH_2), 25.63 (CH_2), 25.01 (2 × CH_2), 21.05. HRMS(EI) *m/z* calcd for $C_{25}H_{31}Cl_2N_3O$, 459.1844; found, 459.1837.

*4.1.21. 4-[(R)-(2,4-dichlorophenyl)(phenyl)methyl]-N-piperidin-1-ylpiperazine-1-carboxamide (**12R**)*

Compound **12R** was prepared from CDI, 1-aminopiperidine and **10b** in 78% yield as white solid by the same procedure as described previously [14]. $[\alpha]_D^{20} = -2.7$ (c 1.0, EtOH), 96% ee (analyzed on an 'Chiralpak AD-H' column, flow rate 1.0 mL/min, temperature 30 °C, $\lambda = 254$ nm, hexane/*i*-PrOH, 97:3 with 0.1% diethylamine, $t_{R\ (minor)} = 30.6$ min, $t_{R\ (major)} = 33.7$). Other physical and chemical data were identical with those described above for the opposite enantiomer **12S**.

*4.1.22. N-cyclohexyl-4-[(R)-(2,4-dichlorophenyl)(p-tolyl)methyl] piperazine-1-carboxamide (**13R**)*

Compound **13R** was prepared from **11b** and cyclohexyl isocyanate in 86% yield as white solid by the same procedure as described previously [14]. $[\alpha]_D^{20} = -4.1$ (c 1.0, EtOH), 95% ee (analyzed on an 'Chiralpak AD-H' column, flow rate 1.0 mL/min, temperature 30 °C, $\lambda = 254$ nm, hexane/*i*-PrOH, 97:3 with 0.1% diethylamine, $t_{R\ (minor)} = 36.2$ min, $t_{R\ (major)} = 41.3$). Other physical and chemical data were identical with those described above for the opposite enantiomer **13S**.

4.2. Biology

4.2.1. Whole cell binding assay

The assay was performed using Chinese hamster ovarian (CHO) cells stably expressing hCB1 or hCB2 receptors (CHO-hCB1/hCB2 cells). Briefly, CHO-hCB1/hCB2 cells were starved in serum-free F12 medium for 3 h and pretreated with different concentrations of test compounds for 10 min before the addition of [^{3}H]-CP-55940. After 3 h incubation at 37 °C, cells were washed 5 times with pre-warmed phosphate-buffered saline (PBS) and lysed by PBS with 1% SDS. The lysed pellets were transferred into 96-well isoplate (PerkinElmer, Waltham, MA, USA) and bound radioligand was measured with 1450 microbeta liquid scintillation luminescence counter (PerkinElmer, Waltham, MA, USA). Assays were performed in triplicate. The *K*i values were analyzed by nonlinear regression analysis performed using the GraphPad Prism 4.0 software (GraphPad Software, San Diego), and calculated by the Cheng–-Prusoff equation: $Ki = IC_{50}/(1 + L/Kd)$.

4.2.2. Calcium concentration assay

Intracellular Ca^{2+} concentration was measured in CHO cells co-transfected with hCB_1 receptors and Gα15/16 (CHO-hCB1-Gα15/16). CHO-hCB1-Gα15/16 cells were loaded with 2 μM fluo-4 AM in Hanks balanced salt solution (HBSS, containing 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$, 0.6 mM $MgSO_4$, 137 mM NaCl, 5.6 mM D-glucose and 250 μM sulfinpyrazone, pH 7.4) at 37 °C for 50 min. After removal of the loading buffer, cells were washed with HBSS once and incubated with 50 μL HBSS containing various concentrations of test compounds or DMSO (negative control, final concentration 1%). After 10 min incubation at room temperature, 25 μL CP55940 were dispensed into the well by FlexStation II micro-plate reader (Molecular Devices, Sunnyvale, CA, USA), and intracellular calcium change was recorded with an excitation wavelength of 485 nm and emission wavelength of 525 nm. Assays were performed in triplicate.

4.2.3. In vivo studies

In vivo pharmacological studies in diet-induced obese (DIO) mice were carried out using male C57BL/6J mice (Shanghai SLAC Laboratory Animals Co., Shanghai, China). All animal care and experimental procedures carried out in accordance with guidelines of the Laboratory Animal Science Center at Shanghai Institute of Materia Medica. Mice were maintained in a 12 h/12 h light–dark cycle with free access to food and water in group housing conditions in a temperature controlled environment (25 °C). To induce obesity, mice were fed a high fat diet (HFD) containing 40.2% sucrose, 23.6% saturated fat (lard), and 23.6% casein, with equal quantities of fiber and minerals as in the mouse regular diet (Shanghai SLAC Laboratory Animals Co., Shanghai, China) from weaning. The HFD supplied 44.6% of calories as fat and 35.4% of calories as carbohydrate comprised of cornstarch and sucrose. Food intake (FI) and body weight (BW) were measured weekly. DIO mice were used at 20–24 weeks of age and conditioned to oral gavage of water for 5 days before experiments.

For 35-day BW assay, DIO mice ($n = 9$–10 mice/group) were caged in plastic cages in group housing conditions and dosed orally with vehicle (0.1% Tween 80) or compounds (10 mg/kg) once per day for 35 days. All DIO mice were exposed to a HFD through the whole experiment. One mouse in vehicle group died on day 32. Mice were dosed 1 h before the dark cycle. FI and BW were measured weekly. Statistical analysis was performed for the BW data using *t*-test.

4.2.4. Pharmacokinetic assays

Male Sprague–Dawley rats were dosed orally at 10 mg/kg after overnight fasting for pharmacokinetic (PK) evaluations. The blood samples were collected at various time points into lithium heparin tubes and centrifuged. The plasma samples were kept at −20 °C until analysis. For brain penetration study, rats were administered orally at 10 mg/kg and sacrificed at 3, 5, and 9 h postdose, respectively. The whole brains and the blood samples were collected and stored at −20 °C. The plasma samples were extracted by protein precipitation and analyzed by LC/MS/MS. The whole rat brain was homogenized, and was extracted by protein precipitation prior to LC/MS/MS analysis.

Acknowledgments

We are indebted to Dr. Oleg Khorev for critical review of this manuscript. This work was supported by 863 Program (the National High Technology Research and Development Program of China, No. 2007AA02Z308), and the National Natural Science Foundation of China (Grants 81001355, 30873162), and Shanghai Commission of Science and Technology (08JC1407701).

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmech.2011.08.030. These data include MOL files and InChiKeys of the most important compounds described in this article.

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