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Original article

Design, syntheses, and characterization of pharmacophore based chemokine receptor CCR5 antagonists as anti prostate cancer agents

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ABSTRACT

Accumulating evidence has shown multiple roles that chemokine receptor CCR5 may play to promote the progression of several types of cancer. The mechanism of such promotion is believed to involve chronic inflammation that creates a microenvironment which enhances tumor survival. Therefore, blocking CCR5 function with an antagonist may provide a novel treatment of cancers such as prostate cancer. Currently, several CCR5 antagonists are available, but all have been optimized for their inhibitory activity on HIV-1 cellular membrane invasion process rather than inhibition on cytoplasmic signaling pathways. Thus, there is need to develop CCR5 antagonists focusing on blockage of CCR5 downstream signaling and inhibition of CCR5 related prostate cancer proliferation and progression. In this report, a pharmacophore analysis was conducted based on docking studies of several known CCR5 antagonists in a CCR5 homology model. A unique structural skeleton for CCR5 antagonist was constructed and functionalized, resulting in a new series of small molecules to be synthesized and characterized. A combination of CCR5 calcium flux inhibition, anti prostate cancer cell proliferation, basal cytotoxicity, and *in vivo* animal model studies were applied to screen the newly synthesized compounds. Results from this study provided a potential lead compound for future CCR5 antagonist development focusing on prostate cancer therapy.

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1. Introduction

Chemokine receptor CCR5, a G protein-coupled receptor (GPCR), has been shown to be a viable target in drug discovery due to its involvement in HIV entry and cancer [1]. In HIV pathogenesis, CCR5 acts as an essential co-receptor for HIV invasion into host cells; whereas in cancer, it provides a pro-inflammatory environment promoting cell invasion and proliferation in several cancers [2–10]. Within the immune system, CCR5 primarily functions through interaction with endogenous small cytokines (chemokines) which include CCL3 (MIP-1α), CCL4 (MIP-1β) and CCL5 (RANTES) [11,12]. Of those chemokines, RANTES expression has been correlated to the progression of several cancers [9,13–15]. Within those cancers, prostate cancer specimens have also been shown to overexpress CCR5 [15]. Importantly, the RANTES induced prostate cancer cell invasion and proliferation can be inhibited by the CCR5 antagonist TAK-779 (Fig. 1) [9]. Therefore, this mechanism of prostate cancer progression and inhibition represents a novel cancer therapy target.

Currently, prostate cancer is the most common non-cutaneous solid cancer in men in the U.S.; in all, approximately one sixth of U.S. men will develop prostate cancer [16]. Several therapies exist for prostate cancer, but are beneficially limited to early stages of the disease. Upon the onset of prostate cancer metastasis no significantly effective therapies exist [17–21]. Therefore, exploiting RANTES-induced cancer cell invasion and proliferation could be a useful therapy to stop the progression of prostate cancer at later stages. In order to do so, CCR5 antagonists specifically targeting prostate cell proliferation and invasion need to be developed.

Several small molecule CCR5 antagonists have been developed as HIV-1 entry inhibitors. All of them have shown high efficacy inhibiting CCR5 mediated virus entry [22–28]. However, there has not been much success in getting them through clinical trials due to toxicity, cardiac side effects, lack of efficacy and bioavailability [1,24–26,29]. In fact, only one of them has been approved by the

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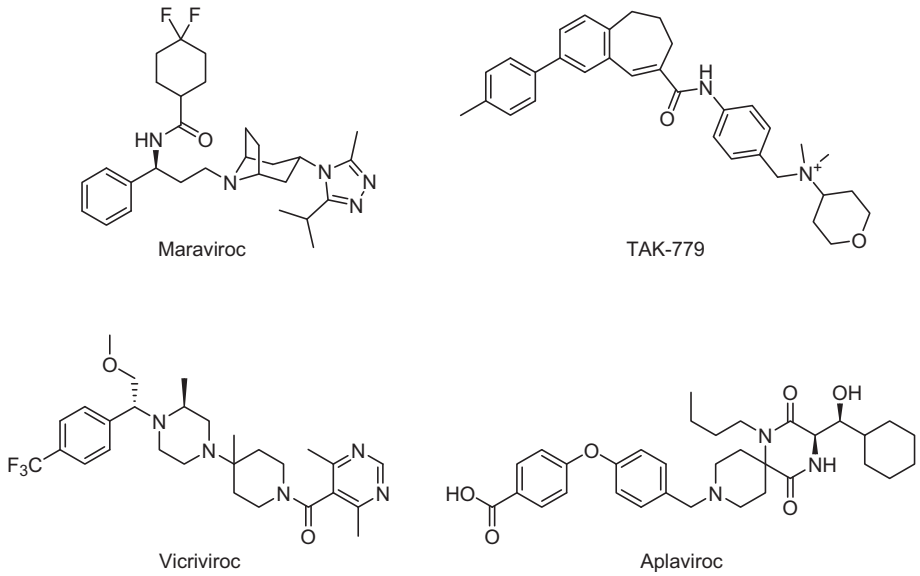


Fig. 1. The chemical structures of several known CCR5 antagonists.

FDA for the treatment of HIV in 2007 (Maraviroc, Fig. 1) [27,28]. Therefore, there is still a need to continue looking for new chemical structures in order to curtail the negative side effects of those compounds. Herein, we designed and synthesized a set of novel CCR5 antagonists based on a shared pharmacophore model of several known CCR5 antagonists obtained through molecular modeling, and tested them for their activity on inhibition of RANTES signaling and cancer cell proliferative, basal cytotoxicity, and tumor growth inhibitory potency.

2. Results and discussion

2.1. Molecular design

To identify the common pharmacophore of known CCR5 antagonists, a conformational analysis of several CCR5 antagonists was first performed to find the lowest energy conformation for each of them. Superimposing the lowest energy conformations of these ligands yielded a general molecular scaffold that each of the structures shared. In all, in the center of the scaffold a secondary or tertiary amino group was connected to an amide moiety at a distance of 5–7 Å, on the right of the scaffold an aromatic moiety is attached to the amide at a distance of 3.5–5.5 Å, and on the left of the scaffold a hydrophobic moiety is linked to the secondary or tertiary amine at a distance of 4.5–6 Å. When looking at the overlaid conformations three-dimensionally the whole scaffold was somehow bent, rather than linear (Fig. 2). Such conformation provided us a starting point of analyzing the possible binding mode of the ligands in the protein.

All the ligands in their lowest energy conformation were docked into a CCR5 homology model constructed based on the crystal structure of CXCR4 [30]. After that, energy minimization and dynamics simulation were conducted to optimize the ligand binding mode. Analyses of the docking modes of four known CCR5 antagonists in the CCR5 homology model (Fig. 3) indicated only a slight change of their final docking mode compared to their original lowest energy conformation, and revealed several commonalities shared among the compounds. All four compounds carried a secondary or tertiary amino group which formed a putative salt bridge with Glu283 (TM7). Additional common interacted included: a hydrophobic moiety fitting into a hydrophobic pocket formed by Ile198 (TM5), Phe109 (TM3), Tyr108 (TM3), and Phe112 (TM3); and all four had an aromatic group binding within an aromatic binding pocket formed by Tyr89 (TM2), Trp86 (TM2), and Trp248 (TM6). Such results further supported the identification of the general pharmacophore of these ligands.

Based upon the shared scaffold of these known CCR5 antagonists and the analysis of their binding modes in the homology model of CCR5, a new series of ligands was proposed as a proof-of-concept to test our observation (Fig. 2). The skeleton chosen was based upon a 1,2,4-trisubstituted benzene ring with its 4-position attached to an N-substituted piperidine ring and provides three different R-group attachment sites, allowing for a library of diverse ligands to be easily synthesized. In detail, R1 group was mainly different sizes of hydrophobic moieties to test the bulkiness influence to the binding pocket; R2 group was introduced as two different aromatic systems; and R3 group was applied to test variety of electronic effects on the hydrophobic moiety.

2.2. Chemistry

A total of 24 compounds with varying substituents were synthesized by applying the route in Fig. 4. The first step involved a Grignard reaction between 1-benzyl-4-piperidone and 1-bromo-4(R_2-substituted)benzene to synthesize **1**. After hydrogenation to

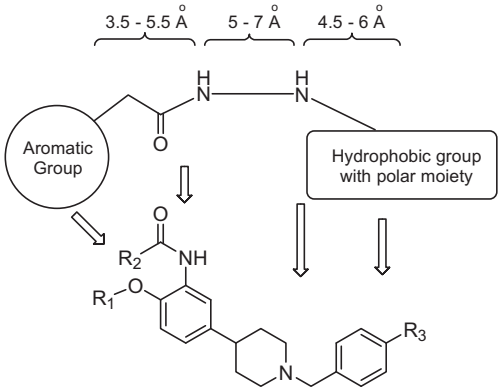


Fig. 2. Proposed pharmacophore of known CCR5 antagonists and molecular scaffold designed to meet its requirements.

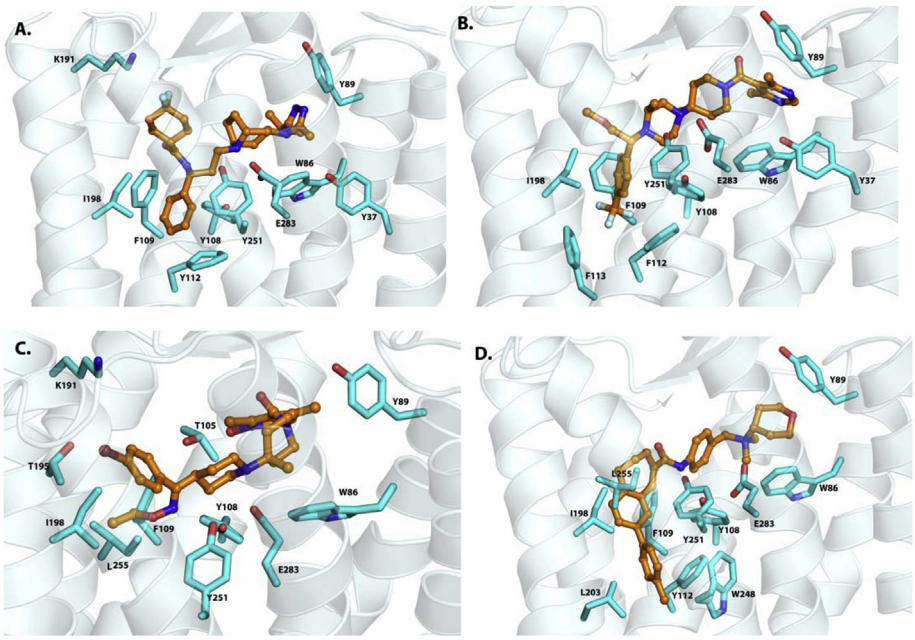


Fig. 3. Docked poses for maraviroc, vicriviroc, SCHC, and TAK779. Ligands are shown in orange balls and sticks, possible interacting residues in cyan sticks.

deprotect the amino group and reduce the alkene group, compound **2** was obtained. Trifluoroacetyl protection of the amino group on the piperidine ring to afford **3** was achieved under typical acetylation conditions. In order to mono-nitrate the ortho-position to synthesize **4**, very mild nitration conditions at low temperature (−60 °C) were applied. The nitro group was then reduced through palladium/carbon catalyzed hydrogenation to give **5**, and compound **5** was coupled to either of the two R_3 substituted carboxylic acids using standard amide coupling conditions to afford **6**. Subsequently, the piperidine amine group was deprotected under basic conditions to yield intermediate **7**, and with another amide coupling reaction and following modification operation the 4-substituted R_1 groups were added to obtain the final target compounds **8** to **31**. All of the final compounds were obtained in reasonable yields and characterized with NMR, IR, MS, and HPLC. Overall, the designed synthetic route allowed a divergent synthesis that was capable of introducing a variety of different substitutions efficiently (Fig. 5).

2.3. Biological screenings

2.3.1. Inhibition of CCR5 agonist RANTES stimulated calcium flux

Inhibition of RANTES induced CCR5 calcium ion mobilization was first applied to test the compounds' ability to bind and inhibit

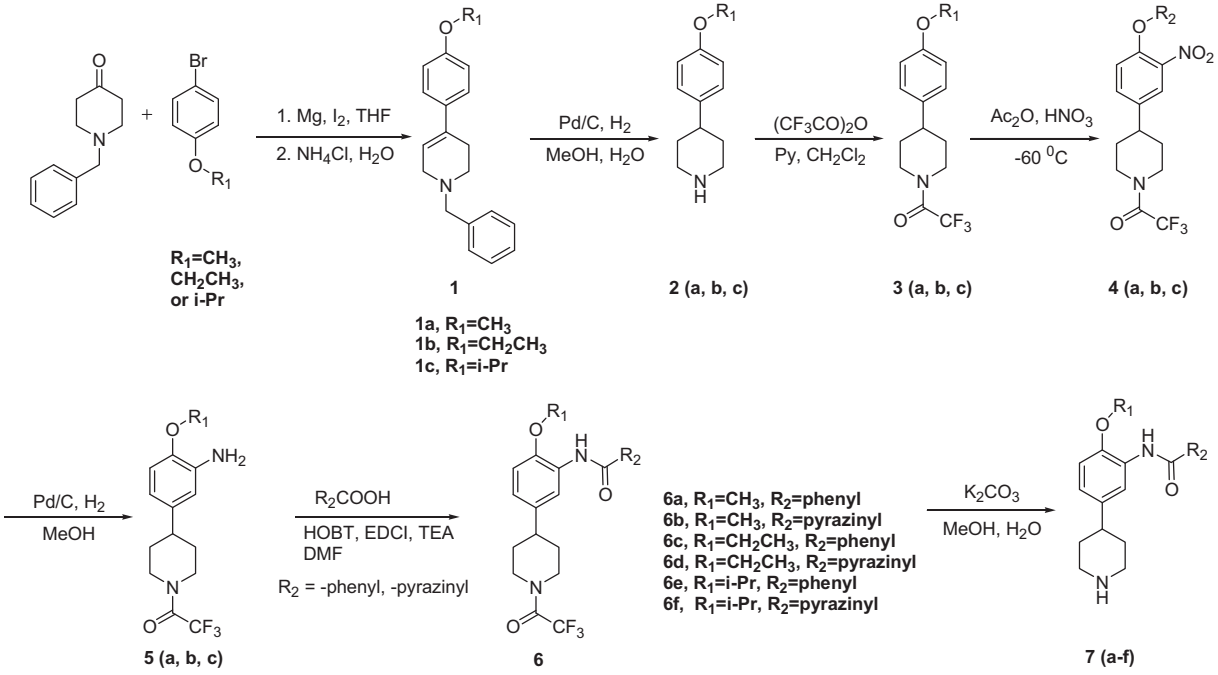


Fig. 4. Synthetic route for the key intermediate compound **7**.

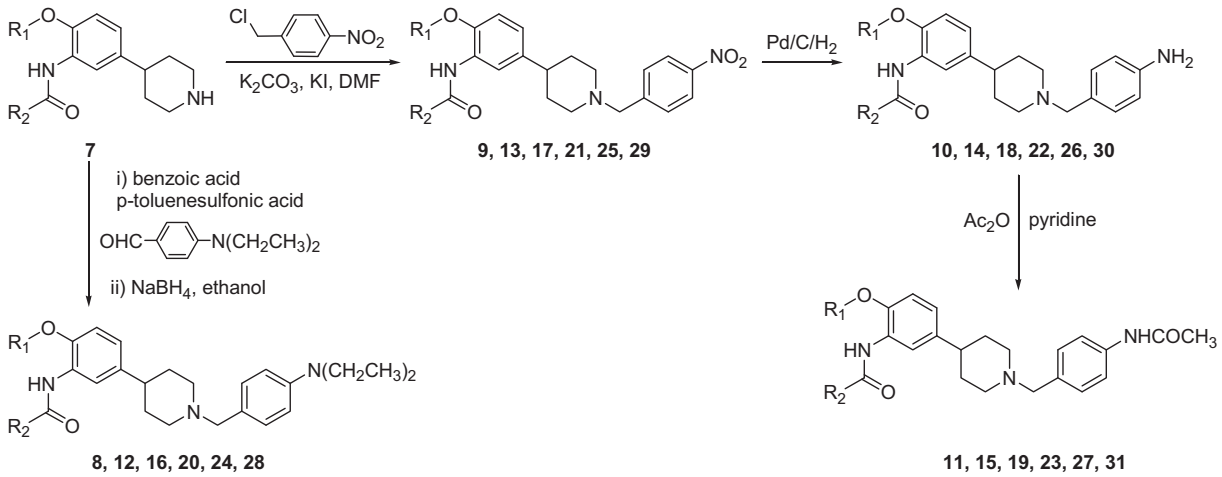
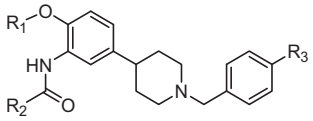


Fig. 5. Syntheses of final compounds **8**–**31**.

CCR5 signaling since it has been shown to correlate well with radioligand binding and functional assays [31,32]. Briefly, CCR5 expressing MOLT-4 cells [33] were transfected with a chimeric G-protein, G_{qi5} [34], and stimulated with RANTES with and without the presence of the newly synthesized compounds, the results were shown in Table 1. First of all, no agonism was observed for any of the new compounds. Second, they all followed a dose-dependent inhibition and acted CCR5 antagonists with moderate inhibitory activity in the calcium flux assay. Several trends were seen: R1 group bulkiness change is not significant enough to influence the CCR5 antagonism; For R2 group, it seemed pyrazinyl substitution in general was more favorable to the inhibitory effect; For R3 groups, it seemed overall nitro group would be better suited. In all, several compounds showed promising CCR5 inhibition below 40 μM, which supported our original molecular design.

Table 1
Synthesized derivatives and their respective CCR5 calcium mobilization inhibition.



Compound	R_1	R_2	R_3	CCR5 inhibition IC_{50} (μM)
8	$-CH_3$	−Phenyl	$-N(CH_2CH_3)_2$	90 ± 22
9	$-CH_3$	−Phenyl	$-NO_2$	28.7 ± 7.9
10	$-CH_3$	−Phenyl	$-NH_2$	77 ± 18
11	$-CH_3$	−Phenyl	$-NCOCH_3$	70.8 ± 8.8
12	$-CH_3$	−Pyrazinyl	$-N(CH_2CH_3)_2$	56 ± 11
13	$-CH_3$	−Pyrazinyl	$-NO_2$	73 ± 29
14	$-CH_3$	−Pyrazinyl	$-NH_2$	38.8 ± 4.0
15	$-CH_3$	−Pyrazinyl	$-NCOCH_3$	59 ± 34
16	$-CH_2CH_3$	−Phenyl	$-N(CH_2CH_3)_2$	64 ± 25
17	$-CH_2CH_3$	−Phenyl	$-NO_2$	28.0 ± 3.1
18	$-CH_2CH_3$	−Phenyl	$-NH_2$	63 ± 26
19	$-CH_2CH_3$	−Phenyl	$-NCOCH_3$	115 ± 48
20	$-CH_2CH_3$	−Pyrazinyl	$-N(CH_2CH_3)_2$	51.8 ± 4.3
21	$-CH_2CH_3$	−Pyrazinyl	$-NO_2$	61 ± 11
22	$-CH_2CH_3$	−Pyrazinyl	$-NH_2$	43.5 ± 5.1
23	$-CH_2CH_3$	−Pyrazinyl	$-NCOCH_3$	47 ± 11
24	$-CH(CH_3)_2$	−Phenyl	$-N(CH_2CH_3)_2$	138 ± 34
25	$-CH(CH_3)_2$	−Phenyl	$-NO_2$	45 ± 15
26	$-CH(CH_3)_2$	−Phenyl	$-NH_2$	66.2 ± 1.8
27	$-CH(CH_3)_2$	−Phenyl	$-NCOCH_3$	92.2 ± 4.9
28	$-CH(CH_3)_2$	−Pyrazinyl	$-N(CH_2CH_3)_2$	61 ± 13
29	$-CH(CH_3)_2$	−Pyrazinyl	$-NO_2$	65.7 ± 1.1
30	$-CH(CH_3)_2$	−Pyrazinyl	$-NH_2$	77.8 ± 1.4
31	$-CH(CH_3)_2$	−Pyrazinyl	$-NCOCH_3$	88.1 ± 6.6

Values shown are mean ± S.E.M. from at least three separate experiments performed in triplicate. The calculated IC_{50} values for CCR5 calcium inhibition were calculated using GraphPad Prism.

2.3.2. Inhibition of prostate cancer cell proliferation assay

Two prostate cancer cell lines, PC-3 and M12, which express both RANTES and CCR5, were chosen as model prostate cancer cell systems [35–37] in order to assess the anti-proliferative activity of these series of compounds. Using a protocol previously described, each compound showed dose-dependent anti-proliferative activity in both cell lines [38]. The results in Table 2 suggested that several of the compounds were capable of inhibiting proliferation in both of the prostate cancer cell lines, including compound **8**, **18**, **19**, **26**, and **27**. For several other compounds they only showed significant inhibitory effect on one cancer cell line, e.g. compound **12**, **16**, **20**, and **24**.

2.3.3. Basal cytotoxicity studies

To ensure the above results were not due the toxicity of the compounds, a basal cytotoxicity assay was conducted. The NIH-3T3 cells used for the assay are mouse fibroblasts that have been used extensively along with neutral red uptake (NRU) to assess compounds' basal cytotoxicity levels [39,40]. In all, several of the compounds that showed high anti-proliferative activity in both M12 and PC-3 were also cytotoxic in NIH-3T3 cells which indicates their observed anti-proliferative activity may be related to their basal cytotoxicity from off-target protein interactions. For example, compound **8** showed an ED_{50} of 12.5 ± 1.6 and 37.2 ± 7.6 μM in M12 and PC-3 respectively, but it showed basal cytotoxicity in NIH-3T3 with a TC_{50} of 28.1 ± 0.6 μM, similar cases for compound **26** and **27**. Therefore, these compounds would not be a good lead due to their similar effects in both cancerous and noncancerous cells. However, two compounds in the series have anti-proliferative activity while displaying low cytotoxicity. Compound **18** showed an ED_{50} of 31.9 ± 0.42 and 43.0 ± 2.3 μM for M12 and PC-3 respectively and displayed a TC_{50} of greater than 80 μM. Similar results were seen for **19**, but due to solubility issues it was not further pursued for the following *in vivo* studies.

2.3.4. In vivo tumor growth inhibition studies

To assess the effect of the potential lead compound **18** on prostate tumor growth *in vivo*, we injected mice subcutaneously with M12 prostate cancer cells M12, followed by treatment with the compound at a dose of 0.3 mg/kg (or saline) once every four

Table 2
Proliferation inhibition in prostate cancer cell lines.

Compound	M12 anti-proliferation ED_{50} (μM)	PC-3 anti-proliferation ED_{50} (μM)	NIH-3T3 cytotoxicity TC_{50} (μM)
8	12.5 ± 1.6	37.2 ± 7.6	28.1 ± 0.6
9	ND	104 ± 3	>100
10	67.6 ± 9.9	53.1 ± 7.5	96 ± 2.2
11	79.9 ± 1.8	60 ± 14	92.2 ± 0.3
12	84.8 ± 5.7	21.6 ± 3.9	63 ± 10
13	ND	ND	>100
14	73.7 ± 8.4	72.3 ± 4.8	>100
15	89.2 ± 2.3	99.2 ± 2.8	95.9 ± 1.5
16	15.9 ± 0.7	86.6 ± 0.8	49.5 ± 1.7
17	122 ± 12	151 ± 34	>100
18	31.9 ± 0.42	43.0 ± 2.3	84 ± 11
19	34.5 ± 1.9	34.7 ± 0.7	87.5 ± 5.9
20	18.7 ± 2.9	77 ± 11	46.9 ± 4.7
21	ND	109 ± 4	>100
22	52.4 ± 8.0	61.7 ± 4.4	76 ± 14
23	58 ± 13	50 ± 19	>100
24	28.7 ± 0.6	70 ± 16	26.5 ± 3.8
25	83.6 ± 8.4	71.2 ± 6.3	>100
26	23.9 ± 3.2	25.1 ± 4.7	27.3 ± 0.87
27	35.6 ± 4.2	41 ± 12	42.0 ± 6.6
28	67 ± 15	75 ± 13	45.3 ± 4.6
29	35.1 ± 1.3	57.2 ± 1.9	56.9 ± 9.4
30	32.7 ± 2.6	ND	>100
31	35.0 ± 1.3	57.2 ± 1.9	94 ± 15

ND denotes that the compound was not tested due to solubility issues. Values shown are mean ± S.E.M. from at least three separate experiments performed in triplicate. The calculated EC_{50} and TC_{50} values for CCR5 calcium inhibition were calculated using GraphPad Prism.

days for total of sixteen days before data analysis. No apparent toxicity of the compounds to the animal, e.g., significant weight loss, was observed during the experiment period. The experiment was terminated when the control group mice were lost due to their fully developed tumors. The results (Fig. 6) showed that compound **18** reduced the subcutaneous growth of M12 tumors in athymic nude mice by over 50% at this dose (P value < 0.05). These results establish compound **18** as our new lead for future molecular design and development of a novel anti prostate cancer agent.

3. Conclusions

In summary, a series of ligands was designed and synthesized as potential CCR5 antagonists targeting the treatment of prostate cancer. As a proof-of-concept, the general skeleton of the molecules was constructed based on known CCR5 antagonists and molecular modeling studies of the CCR5 binding pocket. In CCR5 calcium mobilization inhibition assays all of the compounds acted as antagonists. By using a combination of anti-proliferation assays and basal cytotoxicity assays compounds having the most desirable therapeutic potential were determined. With an ED_{50} of 32 μM and 43 μM in M12 and PC-3 prostate cancer cells and no observable cytotoxicity up to 80 μM, and more impressively an *in vivo* tumor growth inhibition effects observed, compound **18** was defined as our new lead compound. Further modification of this lead will be pursued based upon in depth molecular modeling study and more extensive structure modification on the substitutions of the basic skeleton.

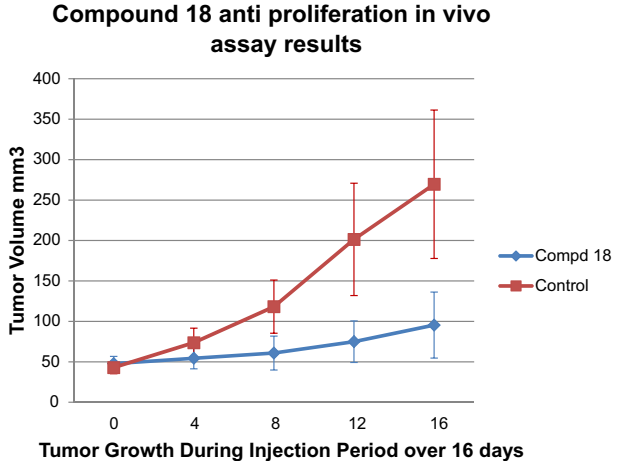


Fig. 6. *In vivo* studies of prostate tumor growth inhibition of the lead compound **18**.

4. Experimental sections

4.1. Chemical syntheses

Melting points were obtained with a Fisher scientific micro melting point apparatus and were uncorrected. Proton (300 MHz) and carbon-13 (75 MHz) nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini-300 "Tesla" spectrometer (300 MHz), with tetramethylsilane as the internal standard. All IR spectra were recorded on a Nicolet Avatar 360 FT-IR Instruments. TLC analyses were carried out on Analtech Uniplate F254 plates. Chromatographic purification was carried out on silica gel columns (160 meshes). Yields were not maximized. For representative spectra, please refer to the Supplementary information.

4.1.1. 1-Bromo-4-isopropoxybenzene

The solution of 4-bromophenol (15.0 g, 86.7 mmol) and isopropyl bromide (16.0 g, 130 mmol) in DMF (50 mL) was added potassium carbonate (14.4 g, 104 mmol). The reaction mixture was heated to reflux overnight. After cooling down, the suspension was filtered, the filtrate was concentrated under vacuum to remove DMF. The residue was partitioned between water (50 mL) and dichloromethane (100 mL). The organic layer was washed with brine, dried over sodium sulfate, concentrated to give 14 g colorless oil, in 75% yield. After dried, the crude product was used for next step without further purification. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.35 (d, J = 7.8 Hz, 2H), 6.76 (d, J = 7.8 Hz, 2H), 4.49 (m, 1H), 1.31 (d, J = 5.7 Hz, 6H).

4.1.2. 1-Benzyl-4-(4-methoxyphenyl)-1,2,3,6-tetrahydropyridine (**1a**)

A solution of methoxybromobenzene (3.74 g, 20 mmol) in THF (20 mL) was added dropwise to a stirred mixture of Magnesium (442 mg, 18.2 mmol) and iodine (catalytic amount) in THF (30 mL) over a 30 min period, then heated to reflux for 30 min. After cooled down, 1-benyl-4-piperidinone (3.78 g, 20 mmol) was added dropwise to the stirred mixture at room temperature over 20 min, and then refluxed for 30 min. After cooling down to room temperature, sat. aq. NH_4Cl (53 mL) and H_2O (25 mL) were added to the mixture, then allowed to stand for a short time. The mixture was added 6 N HCl (53 mL), then refluxed for 3 h. After cooling down, the mixture was concentrated in vacuum to remove THF, the compound was precipitated out from water layer. Filtration gave 3.7 g of compound **1a** in 60% yield. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 12.9 (br, 1H), 7.69 (m, 2H), 7.46 (m, 3H), 7.31 (d, J = 8.1 Hz, 2H), 6.88 (d, J = 8.1 Hz, 2H), 5.88 (s, 1H), 4.25 (m, 2H), 3.98 (m, 1H), 3.80 (s, 3H), 3.58 (m, 2H), 3.19 (m, 2H), 2.65 (m, 1H).

Under a similar procedure, the following two compounds (R1 is ethoxyl, or isoproxyl group) were prepared in 55–70% yield.

4.1.3. 1-Benzyl-4-(4-ethoxyphenyl)-1,2,3,6-tetrahydropyridine (**1b**)

^{1}HNMR ($CDCl_3$, 300 MHz): δ 12.45 (br, 1H), 7.72 (m, 2H), 7.46 (m. 3H), 7.30 (d, J = 8.4 Hz, 2H), 6.86 (d, J = 8.4 Hz, 2H), 5.88 (s, 1H), 4.28 (m, 2H), 4.03 (q, J = 6.9 Hz, 2H), 3.94 (m, 1H), 3.57 (m, 2H), 3.20 (m, 2H), 2.68 (m, 1H), 1.42 (t, J = 6.9 Hz, 3H).

*4.1.4. 1-Benzyl-4-(4-isopropoxyphenyl)-1,2,3,6-tetrahydropyrine (**1c**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 12.49 (br, 1H), 7.71 (m, 2H), 7.46 (m. 3H), 7.29 (d, *J* = 12.0 Hz, 2H), 6.85 (d, *J* = 12.0 Hz, 2H), 5.87 (s, 1H), 4.56 (m, 1H), 4.28 (m, 2H), 3.96 (d, *J* = 13.2 Hz, 1H), 3.62 (m, 1H), 3.50 (d, *J* = 16.2 Hz, 1H), 3.20 (m, 2H), 2.67 (m, 1H), 1.29 (d, *J* = 6.3 Hz, 6H).

*4.1.5. 4-(4-Methoxyphenyl)piperidine (**2a**)*

A solution of compound **1a** (2.3 g, 8.2 mmol) in methanol (100 mL) was hydrogenated in the presence of 5% Pd/C (230 mg) under a H_2 atmosphere (57 psi) at room temperature for 24 h. The mixture was filtered, and the filtrate was concentrated to give compound **2a** (1.8 g, 96% yield). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 9.65 (br, 1H), 9.43 (br, 1H), 7.16 (d, *J* = 8.1 Hz, 2H), 6.86 (d, *J* = 8.1 Hz, 2H), 3.79 (s, 3H), 3.62 (m, 2H), 3.01 (m, 2H), 2.73 (m, 1H), 2.21 (m, 2H), 2.02 (m, 2H).

Under a similar procedure, the following two compounds (R1 is ethoxyl, or isoproxyl group) were prepared in 95–100% yield.

*4.1.6. 4-(4-Ethoxyphenyl)piperidine (**2b**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 9.65 (br, 1H), 7.14 (d, *J* = 7.5 Hz, 2H), 6.84 (d, *J* = 7.5 Hz, 2H), 4.01 (q, *J* = 6.9 Hz, 2H), 3.63 (m, 2H), 3.05 (m, 2H), 2.72 (m, 1H), 2.16 (m, 1H), 2.04 (m, 3H), 1.40 (t, *J* = 6.9 Hz, 3H).

*4.1.7. 4-(4-Isopropoxyphenyl)piperidine (**2c**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 9.27 (br, 1H), 9.07 (br, 1H), 7.13 (d, *J* = 8.4 Hz, 2H), 6.84 (d, *J* = 8.4 Hz, 2H), 4.52 (m, 1H), 3.66 (d, *J* = 11.7 Hz, 2H), 3.02 (m, 2H), 2.72 (m, 1H), 2.23 (m, 2H), 2.04 (d, *J* = 13.5 Hz, 2H), 1.32 (m, 6H).

*4.1.8. 2,2,2-Trifluoro-1-(4-(4-methoxyphenyl)piperidin-1-yl) ethanone (**3a**)*

To a solution of compound **2a** (1.8 g, 8 mmol) in dichloromethane (100 mL) and pyridine (1.4 g, 17.6 mmol), trifluoroacetic anhydride (1.85 g, 8.8 mmol) was added dropwise. After stirred at room temperature overnight, the resulting suspension was washed with 1 N HCl (25 mL × 2) and brine, dried with Na_2SO_4. The CH_2Cl_2 solution was filtered, the filtrate was concentrated to give compound **3a** (2.07 g, 90% yield). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.11 (d, *J* = 8.7 Hz, 2H), 6.86 (d, *J* = 8.7 Hz, 2H), 4.68 (d, *J* = 12.0 Hz, 1H), 4.12 (d, *J* = 12.0 Hz, 1H), 3.80 (s, 3H), 3.23 (m, 1H), 2.80 (m, 2H), 1.95 (d, *J* = 12.3 Hz, 2H), 1.69 (m, 2H).

Under a similar procedure, the following two compounds (R1 is ethoxyl, or isoproxyl group) were prepared in comparable yield.

*4.1.9. 2,2,2-Trifluoro-1-(4-(4-ethoxyphenyl)piperidin-1-yl) ethanone (**3b**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.09 (d, *J* = 8.7 Hz, 2H), 6.84 (d, *J* = 8.7 Hz, 2H), 4.68 (d, *J* = 13.2 Hz, 1H), 4.11 (d, *J* = 14.1 Hz, 1H), 4.01 (q, *J* = 7.2 Hz, 2H), 3.22 (m, 1H), 2.77 (m, 2H), 1.95 (d, *J* = 14.1 Hz, 2H), 1.70 (m, 2H), 1.40 (t, *J* = 7.2 Hz, 3H).

*4.1.10. 2,2,2-Trifluoro-1-(4-(4-isopropoxyphenyl)piperidin-1-yl)-ethanone (**3c**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.09 (d, *J* = 8.7 Hz, 2H), 6.84 (d, *J* = 8.7 Hz, 2H), 4.67 (m, 1H), 4.53 (m, 1H), 4.13 (d, *J* = 13.8 Hz, 1H), 3.23 (m, 1H), 2.77 (m, 2H), 1.96 (d, *J* = 12.9 Hz, 2H), 1.67 (qd, *J* = 4.2 Hz, 13.2 Hz, 2H), 1.33 (m, 6H).

*4.1.11. 2,2,2-Trifluoro-1-(4-(4-methoxy-3-nitrophenyl)piperidin-1-yl)ethanone(**4a**)*

To a solution of compound **3a** (1.5 g, 5.22 mmol) in Ac_2O (40 mL), on a dry ice-acetone bath, nitric acid (4.75 g, 69%, 52 mmol) was added dropwise. The reaction mixture was stirred at −30 °C for 6 h. Then the mixture was poured into ice (50 mL). The water phase was extracted with CH_2Cl_2 (50 mL × 3), washed with brine, dried, and concentrated to yield 1.7 g of compound **4a** (in 98% yield). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.38 (d, *J* = 9.0 Hz, 1H), 7.38 (d, *J* = 9.0 Hz, 1H), 7.03 (d, *J* = 9.0 Hz, 1H), 4.72 (d, *J* = 14.1 Hz, 1H), 4.15 (d, *J* = 14.1 Hz, 1H), 3.95 (s, 3H), 3.25 (m, 1H), 2.86 (m, 2H), 2.00 (d, *J* = 12.9 Hz, 2H), 1.69 (m, 2H).

Under a similar procedure, the following two compounds (R1 is ethoxyl, or isoproxyl group) were prepared in comparable yields.

*4.1.12. 2,2,2-Trifluoro-1-(4-(4-ethoxy-3-nitrophenyl)piperidin-1-yl) ethanone (**4b**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.66 (d, *J* = 2.4 Hz, 1H), 7.34 (dd, *J* = 2.4, 8.4 Hz, 1H), 7.03 (d, *J* = 8.4 Hz, 1H), 4.70 (m. 2H), 4.17 (q, *J* = 6.6 Hz, 3H), 3.24 (m, 1H), 2.83 (m, 2H), 2.02 (d, *J* = 13.8 Hz, 2H), 1.67 (m, 2H), 1.47 (t, *J* = 6.6 Hz, 3H).

*4.1.13. 2,2,2-Trifluoro-1-(4-(4-isopropoxy-3-nitrophenyl)piperidin-1-yl)ethanone (**4c**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.60 (d, *J* = 2.1 Hz, 1H), 7.30 (dd, *J* = 2.4 Hz, 8.4 Hz, 1H), 7.03 (d, *J* = 8.4 Hz, 1H), 4.70 (m, 1H), 4.64 (q, *J* = 6.0 Hz, 1H), 4.15 (m, 1H), 3.24 (m, 1H), 2.83 (m, 2H), 1.99 (m, 2H), 1.70 (m, 2H), 1.38 (d, *J* = 6.3 Hz, 6H).

*4.1.14. 1-(4-(3-Amino-4-methoxyphenyl)piperidin-1-yl)-2,2,2-trifluoroethanone (**5a**)*

A solution of compound **4a** (1.0 g, 3 mmol) in methanol (50 mL) was hydrogenated in the presence of 5% Pd/C (100 mg) under a H_2 atmosphere (57 psi) at room temperature for 24 h. The mixture was filtered, and the filtrate was concentrated to give compound **5a** as gray foam (0.9 g, 99% yield). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 6.72 (d, *J* = 6.9 Hz, 1H), 6.55 (m, 2H), 4.66 (d, *J* = 12.0 Hz, 1H), 4.10 (d, *J* = 13.5 Hz, 1H), 3.83 (s, 3H), 3.79 (m, 2H), 3.20 (t, *J* = 12.9 Hz, 1H), 2.82 (t, *J* = 12.6 Hz, 1H), 2.68 (m, 1H), 1.93 (t, *J* = 13.5 Hz, 2H), 1.64 (m, 2H).

Under a similar procedure, the following two compounds (R1 is ethoxyl, or isoproxyl group) were prepared in comparable yields.

*4.1.15. 1-(4-(3-Amino-4-ethoxyphenyl)piperidin-1-yl)-2,2,2-trifluoroethanone (**5b**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.4 (br, 1H), 7.52 (s, 1H), 7.16 (d, *J* = 10.9 Hz, 1H), 6.92 (q, *J* = 8.1 Hz, 1H), 4.68 (d, *J* = 12.0 Hz, 1H), 4.13 (q, *J* = 7.2 Hz, 1H), 3.21 (d, *J* = 13.5 Hz, 1H), 2.82 (m, 2H), 1.95 (d, *J* = 12.9 Hz, 2H), 1.68 (m, 2H), 1.46 (t, *J* = 7.2 Hz, 3H).

*4.1.16. 1-(4-(3-Amino-4-isopropoxyphenyl)piperidin-1-yl)-2,2,2-trifluoroethanone (**5c**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 7.55 (d, *J* = 2.1 Hz, 1H), 7.13 (dd, *J* = 2.1 Hz, 8.7 Hz, 1H), 6.92 (d, *J* = 8.4 Hz, 1H), 4.64 (m, 2H), 4.12 (d, *J* = 12.6 Hz, 1H), 3.20 (m, 1H), 2.80 (m, 2H), 1.95 (d, *J* = 13.2 Hz, 2H), 1.65 (m, 2H), 1.39 (d, *J* = 5.7 Hz, 6H).

*4.1.17. N-(2-Methoxy-5-(1-(2,2,2-trifluoroacetyl)piperidin-4-yl) phenyl)benzamide (**6a**)*

On an ice-water bath, a solution of benzoic acid (650 mg, 5.5 mmol) (or 2-pyrazinecarboxylic acid) in DMF (2 mL), was added EDC (750 mg, 4 mmol), HOBt (535 mg, 4 mmol), 4 Å molecular sieves, and TEA (800 mg, 8 mmol) under N_2 protection. After 20 min, a solution of compound **5a** (800 mg, 2.65 mmol) in DMF (2 mL) was added dropwise. After stirring at r.t. overnight, the resulting mixture was filtered through celite. The filtrate was concentrated in vacuum to remove DMF. The residue was added brine, taken up with CH_2Cl_2 (50 mL × 3). The organic layer was washed with brine, dried, and concentrated. The residue was purified with chromatography (Hexane/EtOAc: 5/1) to give 800 mg compound **6a** in 74% yield. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.59 (s, 1H),

8.48 (s, 1H), 7.90 (d, J = 7.2 Hz, 2H), 7.52 (m, 2H), 6.89 (d, J = 8.1 Hz, 1H), 6.86 (d, J = 8.1 Hz, 1H), 4.68 (d, J = 13.2 Hz, 1H), 4.13 (d, J = 13.8 Hz, 1H), 3.91 (s, 3H), 3.23 (t, J = 12.9 Hz, 1H), 2.84 (m, 2H), 2.01 (d, J = 13.2 Hz, 2H), 1.73 (m, 2H).

Under a similar procedure, the following compounds (R1 is methyl, ethoxyl, or isoproxyl group; R2 is phenyl, or pyrazinyl group) were prepared in 74–85% yield.

*4.1.18. N-(2-Methoxy-5-(1-(2,2,2-trifluoroacetyl)piperidin-4-yl) phenyl)pyrazine-2-carboxamide (**6b**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.29 (br, 1H), 9.50 (s. 1H), 8.81 (d, J = 2.4 Hz, 1H), 8.63 (dd, J = 1.5, 2.4 Hz, 1H), 8.52 (d, J = 2.4 Hz, 1H), 6.93 (m, 2H), 4.70 (m, 1H), 4.14 (d, J = 12.6 Hz, 1H), 3.96 (s, 3H), 3.26 (m, 1H), 2.85 (m, 2H), 2.02 (d, J = 13.5 Hz, 2H), 1.73 (m, 2H).

*4.1.19. N-(2-Ethoxy-5-(1-(2,2,2-trifluoroacetyl)piperidin-4-yl) phenyl)benzamide (**6c**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.66 (br, 1H), 8.49 (s, 1H), 7.89 (d, J = 7.2 Hz, 2H), 7.54 (m, 3H), 6.87 (m, 2H), 4.69 (d, J = 13.2 Hz, 1H), 4.13 (m, 3H), 3.23 (t, J = 12.9 Hz, 2H), 2.85 (m, 2H), 2.00 (d, J = 13.5 Hz, 2H), 1.73 (m, 2H), 1.49 (t, J = 6.6 Hz, 3H).

*4.1.20. N-(2-Ethoxy-5-(1-(2,2,2-trifluoroacetyl)piperidin-4-yl) phenyl)pyrazine-2-carboxamide (**6d**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.36 (br, 1H), 9.50 (d, J = 1.5 Hz, 1H), 8.79 (d, J = 2.4 Hz, 1H), 8.62 (m, 1H), 8.51 (d, J = 1.5 Hz, 1H), 6.91 (m, 2H), 4.69 (m, 1H), 4.18 (q, J = 6.9 Hz, 2H), 4.13 (m, 1H), 3.25 (td, J = 3.3 Hz, 14.4 Hz, 1H), 2.84 (m, 2H), 2.02 (d, J = 13.2 Hz, 2H), 1.72 (m, 2H), 1.54 (t, J = 6.9 Hz, 3H).

*4.1.21. N-(2-Isopropoxy-5-(1-(2,2,2-trifluoroacetyl)piperidin-4-yl) phenyl)benzamide (**6e**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.69 (br, 1H), 8.50 (s, 1H), 7.88 (m, 2H), 7.54 (m, 3H), 6.88 (s, 2H), 4.72 (d, J = 12.3 Hz, 1H), 4.64 (m, 1H), 4.14 (d, J = 13.5 Hz, 1H), 3.23 (m, 1H), 2.83 (m, 2H), 2.01 (m, 2H), 1.75 (m, 2H), 1.40 (d, J = 6.0 Hz, 6H).

*4.1.22. N-(2-Isopropoxy-5-(1-(2,2,2-trifluoroacetyl)piperidin-4-yl) phenyl)pyrazine-2-carboxamide (**6f**)*

^{1}HNMR (DMSO-d_6, 300 MHz): δ 10.41 (br, 1H), 9.50 (d, J = 1.2 Hz, 1H), 8.80 (dd, J = 1.2, 2.4 Hz, 1H), 8.63 (dd, J = 1.2, 2.4 Hz, 1H), 8.52 (s, 1H), 6.91 (s, 2H), 4.70 (d, J = 13.2 Hz, 1H), 4.60 (q, J = 6.0 Hz, 1H), 4.14 (d, J = 13.2 Hz, 1H), 3.25 (t, J = 11.4 Hz, 1H), 2.84 (m, 2H), 2.02 (d, J = 13.8 Hz, 2H), 1.77 (m, 2H), 1.43 (dd, J = 1.2, 6.9 Hz, 6H).

*4.1.23. N-(2-Methoxy-5-(piperidin-4-yl)phenyl)benzamide(**7a**)*

To the solution of compound **6a** (0.70 g, 1.72 mmol) in CH_3OH (40 mL) and H_2O (2.4 mL) was added potassium carbonate (1.23 g, 8.94 mmol). The reaction mixture was heated to reflux for 2 h. After the solvent was evaporated, water was added to the residue. The water layer was basified with NH_4OH (20 mL), then extracted with $CHCl_3$ (40 mL × 4). The organic phase was washed with brine, dried, and concentrated to give the product (510 mg oil, in 96% yield). ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.56 (s, 1H), 8.47 (d, J = 2.1 Hz, 1H), 7.90 (m, 2H), 7.53 (m, 2H), 6.95 (dd, J = 2.1, 8.4 Hz, 1H), 6.86 (d, J = 8.4 Hz, 1H), 3.92 (s, 3H), 3.21 (d, J = 11,7 Hz, 2H), 2.76 (t, J = 12,0 Hz, 2H), 2.66 (m, 1H), 1.88 (d, J = 15.6 Hz, 2H), 1.68 (m, 2H).

Under a similar procedure, the following compounds (R1 is methyl, ethoxyl, or isoproxyl group; R2 is phenyl, or pyrazinyl group) were prepared in comparable yields.

*4.1.24. N-(2-Methoxy-5-(piperidin-4-yl)phenyl)pyrazine-2-carboxamide (**7b**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.27 (br, 1H), 9.50 (d, J = 1.2 Hz, 1H), 8.79 (d, J = 2.1 Hz, 1H), 8.62 (dd, J = 1.2, 2.1 Hz, 1H), 8.52 (d, J = 2.1 Hz, 1H), 6.98 (dd, J = 2.1, 8.4 Hz, 1H), 6.89 (d, J = 8.4 Hz, 1H), 3.95 (s, 3H), 3.18 (m, 2H), 2.70 (m, 3H), 1.87 (d, J = 12.6 Hz, 2H), 1.65 (m, 2H).

*4.1.25. N-(2-Ethoxy-5-(piperidin-4-yl)phenyl)benzamide (**7c**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.64 (br, 1H), 8.48 (d, J = 2.1 Hz, 1H), 7.89 (d, J = 7.8 Hz, 2H), 7.53 (m, 3H), 6.99 (d, J = 8.7 Hz, 1H), 6.85 (d, J = 8.7 Hz, 1H), 4.13 (q, J = 7.2 Hz, 2H), 3.18 (d, J = 12.3 Hz, 2H), 2.70 (m, 3H), 1.86 (d, J = 12.0 Hz, 2H), 1.66 (td, J = 3.9 Hz, 12.0 Hz, 2H), 1.48 (t, J = 7.2 Hz, 2H).

*4.1.26. N-(2-Ethoxy-5-(piperidin-4-yl)phenyl)pyrazine-2-carboxamide (**7d**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.34 (s, 1H), 9.50 (s, 1H), 8.78 (m, 1H), 8.61 (dd, J = 1.5 Hz, 2.4 Hz, 1H), 8.51 (d, J = 2.1 Hz, 1H), 6.95 (dd, J = 2.1 Hz, 8.1 Hz, 1H), 6.88 (d, J = 8.1 Hz, 1H), 4.16 (q, J = 7.2 Hz, 2H), 3.19 (d, J = 12.3 Hz, 2H), 2.75 (td, J = 2.1 Hz, 12.0 Hz, 2H), 2.65 (m, 1H), 1.87 (d, J = 14.6 Hz, 2H), 1.65 (m, 2H), 1.51 (t, J = 7.2 Hz, 3H).

*4.1.27. N-(2-Isopropoxy-5-(piperidin-4-yl)phenyl)benzamide (**7e**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.67 (s, 1H), 8.49 (s, 1H), 7.90 (m, 2H), 7.53 (m, 3H), 6.91 (m, 2H), 4.61 (q, J = 6.3 Hz, 1H), 3.21 (d, J = 12.9 Hz, 2H), 2.76 (t, J = 12.3 Hz, 2H), 2.65 (m, 1H), 1.75 (m, 2H), 1.66 (m, 2H), 1.39 (d, J = 6.3 Hz, 6H).

*4.1.28. N-(2-Isopropoxy-5-(piperidin-4-yl)phenyl)pyrazine-2-carboxamide (**7f**)*

^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.39 (br, 1H), 9.50 (d, J = 1.5 Hz, 1H), 8.79 (d, J = 2.1 Hz, 1H), 8.63 (m, 2H), 6.96 (dd, J = 2.1, 8.4 Hz, 1H), 6.91 (d, J = 8.4 Hz, 1H), 4.59 (q, J = 6.3 Hz, 1H), 3.22 (d, J = 11.7 Hz, 2H), 2.76 (t, J = 12.0 Hz, 2H), 2.66 (m, 1H), 1.90 (m, 2H), 1.70 (m, 2H), 1.42 (dd, J = 1.8, 6.3 Hz, 6H).

4.2. Preparation of the final compounds

4.2.1. Procedure A

*4.2.1.1. N-(5-(1-(4-(Diethylamino)benzyl)piperidin-4-yl)-2-methoxyphenyl)benzamide (**8**).* A mixture of compound **7a** (180 mg, 0.58 mmol), benzoic acid (206 mg, 1.16 mmol), 4-(diethylamino)-benzaldehyde (142 mg, 1.16 mmol), and a trace of *p*-toluenesulfonic acid in toluene (40 mL), ethanol (40 mL) was refluxed for 24 h, using a Dean–Stark trap for removal of water. The solution was concentrate to 15 mL at 1 atm. Anhydrous ethanol (40 mL), $NaBH_4$ (66 mg, 1.74 mmol) and 4 Å molecular sieves was added, and the resulting solution was stirred under N_2 for 8 h. The mixture was diluted with MeOH (40 mL) and filtered. The residue obtained after removal of solvent was partitioned between CH_2Cl_2 and NH_4OH. The combined CH_2Cl_2 extracts was washed with brine, dried, and concentrated. The residue was purified with chromatography (CH_2Cl_2/MeOH: 50/1) to give 80 mg product **8** in 37% yield. M.p.: 177–179 °C. IR (KBr, cm^{-1}) ν_{max}: 3447, 2932, 1670, 1536, 1025, 699. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.54 (s, 1H), 8.45 (s, 1H), 7.88 (d, J = 6.9 Hz, 2H), 7.50 (m, 3H), 7.18 (d, J = 8.7 Hz, 2H), 6.95 (d, J = 8.7 Hz, 1H), 6.85 (d, J = 8.7 Hz, 1H), 6.64 (d, J = 8.7 Hz, 2H), 3.90 (s, 3H), 3.48 (s, 2H), 3.34 (q, J = 6.8 Hz, 4H), 3.05 (d, J = 11.4 Hz, 2H), 2.51 (m, 1H), 2.05 (m, 2H), 1.83 (m, 4H), 1.16 (t, J = 6.9 Hz, 6H). ^{13}CNMR (100 MHz, CD_3OD) δ: 168.27, 150.93, 137.18, 136.02, 134.51, 134.51, 133.13, 133.13, 129.87, 129.86, 129.85, 128.40, 128.39, 128.38, 128.35, 124.63, 121.94, 112.26, 112.25, 40.35, 33.33, 31.87, 31.86, 11.82. MS (ESI) m/z: 472.3 $(M + H)^+$.

Procedure A was applied to prepare the following compounds, **12**, **16**, **20**, **24**, **28**.

*4.2.1.2. N-(5-(1-(4-(Diethylamino)benzyl)piperidin-4-yl)-2-methoxyphenyl)pyrazine-2-carboxamide (**12**).* M.p.: 200–202 °C. IR

(KBr, cm^{-1}) ν_{max}: 3354, 2945, 1670, 1545, 1202, 691. ^{1}HNMR (300 MHz, CD_3OD): δ 8.90 (br, 1H), 8.42 (m, 3H), 7.92 (d, J = 6.9 Hz, 2H), 7.83 (d, J = 6.9 Hz, 2H), 7.05 (m, 2H), 4.51 (s, 3H), 3.34 (m, 2H), 2.93 (m, 2H), 2.14 (m, 4H), 1.90 (m, 6H). ^{13}CNMR (100 MHz, DMSO) δ: 160.29, 148.17, 148.16, 147.29, 144.00, 143.55, 143.40, 136.58, 133.03, 133.00, 133.00, 126.39, 126.38, 123.70, 122.52, 117.69, 111.18, 58.20, 56.40, 56.17, 56.15, 51.53, 51.53, 38.40, 29.77, 29.76, 10.10, 10.09. MS (ESI) m/z: 446.3 $(M + H)^+$.

*4.2.1.3. N-(5-(1-(4-(Diethylamino)benzyl)piperidin-4-yl)-2-ethoxyphenyl)benzamide (**16**).* M.p.: 182–184 °C. IR (KBr, cm^{-1}) ν_{max}: 3454, 2976, 1672, 1536, 1266, 697. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.62 (s, 1H), 8.48 (s, 1H), 7.89 (d, J = 6.9 Hz, 2H), 7.52 (m, 3H), 7.17 (d, J = 8.1 Hz, 2H), 6.92 (d, J = 8.1 Hz, 1H), 6.82 (d, J = 8.1 Hz, 1H), 6.64 (d, J = 8.1 Hz, 2H), 4.11 (q, J = 7.2 Hz, 2H), 3.44 (s, 2H), 3.30 (q, J = 6.9 Hz, 4H), 3.02 (d, J = 11.1 Hz, 2H), 2.49 (m, 1H), 2.03 (m, 2H), 1.81 (m, 4H), 1.46 (t, J = 6.9 Hz, 3H), 1.15 (t, J = 6.9 Hz, 6H). ^{13}CNMR (100 MHz, $CDCl_3$) δ: 165.20, 146.23, 135.99, 135.22, 134.50, 133.51, 131.84, 129.00, 128.89, 128.87, 128.85, 127.75, 127.10, 126.94, 126.93, 126.90, 121.05, 119.17, 114.42, 64.51, 53.50, 53.48, 50.00, 46.10, 46.09, 40.05, 34.90, 30.27, 30.27, 14.91, 14.91. MS (ESI) m/z: 486.4 $(M + H)^+$.

*4.2.1.4. N-(5-(1-(4-(Diethylamino)benzyl)piperidin-4-yl)-2-ethoxyphenyl)pyrazine-2-carboxamide (**20**).* M.p.: 192–194 °C. IR (KBr, cm^{-1}) ν_{max}: 3430, 2950, 1687, 1535, 1018, 701. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.33 (s, 1H), 9.49 (d, J = 1.2 Hz, 1H), 8.77 (d, J = 2.4 Hz, 1H), 8.60 (dd, J = 1.5, 2.4 Hz, 1H), 8.50 (d, J = 2.1 Hz, 1H), 7.17 (d, J = 8.4 Hz, 2H), 6.99 (dd, J = 2.1 Hz, 8.4 Hz, 1H), 6.85 (d, J = 8.4 Hz, 1H), 6.64 (d, J = 8.4 Hz, 2H), 4.14 (q, J = 7.2 Hz, 2H), 3.45 (s, 2H), 3.34 (q, J = 7.2 Hz, 4H), 3.04 (d, J = 8.1 Hz, 2H), 2.52 (m, 1H), 2.04 (m, 2H), 1.83 (m, 4H), 1.48 (t, J = 6.9 Hz, 3H), 1.16 (t, J = 6.9 Hz, 6H). ^{13}CNMR (100 MHz, CD_3OD) δ: 169.00, 149.65, 148.00, 144.91, 144.00, 137.42, 136.10, 135.05, 135.03, 128.26, 124.53, 124.51, 124.50, 119.41, 119.40, 113.09, 113.00, 60.80, 56.82, 56.80, 54.61, 54.38, 54.36, 40.44, 31.92, 31.90, 30.00, 10.84, 10.83. MS (ESI) m/z: 488.3 $(M + H)^+$.

*4.2.1.5. N-((5-(1-(4-(Diethylamino)benzyl)piperidin-4-yl)-2-isopropoxy)phenyl)benzamide (**24**).* M.p.: 168–170 °C. IR (KBr, cm^{-1}) ν_{max}: 3430, 2977, 1670, 1533, 1111, 702. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.65 (s, 1H), 8.47 (s, 1H), 7.88 (m, 2H), 7.51 (m, 3H), 7.17 (d, J = 8.1 Hz, 2H), 6.89 (d, J = 8.4 Hz, 1H), 6.85 (d, J = 8.4 Hz, 1H), 6.64 (d, J = 8.1 Hz, 2H), 4.58 (m, 1H), 3.44 (s, 2H), 3.36 (q, J = 7.2 Hz, 4H), 3.03 (d, J = 10.8 Hz, 2H), 2.51 (m, 1H), 2.04 (m, 2H), 1.82 (m, 4H), 1.39 (d, J = 5.7 Hz, 6H), 1.16 (t, J = 6.9 Hz, 6H). ^{13}CNMR (100 MHz, $CDCl_3$) δ: 165.10, 145.43, 139.20, 135.89, 134.14, 134.13, 131.85, 131.84, 128.96, 128.90, 128.88, 128.82, 126.91, 126.90, 123.84, 121.17, 120.01, 119.13, 113.20, 71.82, 59.73, 53.60, 53.59, 53.32, 53.30, 39.93, 30.33, 22.33, 22.30, 20.29, 10.51, 10.50. MS (ESI) m/z: 500.4 $(M + H)^+$.

*4.2.1.6. N-(5-(1-(4-(Diethylamino)benzyl)piperidin-4-yl)-2-isopropoxyphenyl)pyrazine-2-carboxamide (**28**).* M.p.: 156–158 °C. IR (KBr, cm^{-1}) ν_{max}: 3567, 3349, 2977, 1685, 1253, 1019, 820. ^{1}HNMR (DMSO, 300 MHz): δ 10.30 (s, 1H), 9.34 (d, J = 1.2 Hz, 1H), 8.98 (m, 1H), 8.84 (dd, J = 1.2, 2.4 Hz, 1H), 8.40 (s, 1H), 7.86 (m, 2H), 7.53 (m, 2H), 7.14 (d, J = 8.4 Hz, 1H), 6.99 (d, J = 8.4 Hz, 1H), 4.68 (q, J = 5.7 Hz, 1H), 4.08 (s, 2H), 3.95 (m, 4H), 3.43 (m, 2H), 3.06 (m, 2H), 2.78 (m, 1H), 1.99 (m, 4H), 1.36 (d, J = 5.7 Hz, 6H) 1.08 (t, J = 6.9 Hz, 6H). ^{13}CNMR (100 MHz, DMSO) δ: 160.14, 148.16, 145.28, 144.10, 143.96, 143.52, 143.50, 136.85, 136.20, 132.99, 132.98, 129.97, 127.78, 122.90, 122.41, 117.48, 114.32, 71.69, 52.25, 52.23, 51.47, 51.45, 38.47, 29.75, 29.73, 21.93, 21.77, 9.70, 9.68. MS (ESI) m/z: 502.4 $(M + H)^+$.

4.2.2. Procedure B

*4.2.2.1. N-(5-(1-(4-Nitrobenzyl)piperidin-4-yl)-2-methoxyphenyl) benzamide (**9**).* To the solution of compound **7a** (400 mg, 1.28 mmol) in DMF (12 mL), potassium carbonate (265 mg, 1.92 mmol), a trace amount of potassium iodine, 4-nitrobenzyl chloride (264 mg, 1.54 mmol) was added. Then the mixture was stirred overnight at r.t. After removal of DMF in vacuum, the residue was purified with chromatography (CH_2Cl_2/MeOH: 100/1) to afford 550 mg product **9** in 96% yield. M.p.: 218–220 °C. IR (KBr, cm^{-1}) ν_{max}: 3400, 2900, 1665, 1534, 1348, 1026, 701. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.57 (br, 1H), 8.50 (d, J = 2.1 Hz, 1H), 8.19 (d, J = 9.0 Hz, 2H), 7.90 (d, J = 6.9 Hz, 2H), 7.53 (m, 5H), 6.95 (dd, J = 2.1, 8.4 Hz, 1H), 6.86 (d, J = 8.4 Hz, 1H), 3.91 (s, 3H), 3.62 (s, 2H), 2.96 (d, J = 13.2 Hz, 2H), 2.54 (m, 1H), 2.13 (m, 3H), 1.85 (m, 3H). ^{13}CNMR (75 MHz, DMSO-d_6) δ: 164.90, 150.06, 148.09, 137.23, 136.02, 134.45, 132.91, 132.43, 131.60, 128.47, 128.46, 127.38, 127.36, 126.81, 123.61, 123.60, 123.40, 122.38, 111.49, 57.72, 55.85, 55.84, 51.79, 38.10, 29.74, 29.73. MS (ESI) m/z: 446.3 $(M + H)^+$.

Procedure B was applied to prepare compounds **13**, **17**, **21**, **25**, **29** with similar yields.

*4.2.2.2. N-(5-(1-(4-Nitrobenzyl)piperidin-4-yl)-2-methoxyphenyl) pyrazine-2-carboxamide (**13**).* M.p.: 238–240 °C. IR (KBr, cm^{-1}) ν_{max}: 3355, 2987, 1680, 1351, 1020, 696. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.28 (br, 1H), 9.50 (d, J = 1.2 Hz, 1H), 8.80 (d, J = 2.1 Hz, 1H), 8.62 (dd, J = 1.5, 2.1 Hz, 1H), 8.54 (d, J = 1.8 Hz, 1H), 8.19 (dd, J = 2.1, 12.0 Hz, 2H), 7.55 (d, J = 12.0 Hz, 2H), 6.98 (dd, J = 2.4, 8.4 Hz, 1H), 6.88 (d, J = 8.4 Hz, 1H), 3.95 (s, 3H), 3.62 (s, 2H), 2.96 (m, 2H), 2.55 (m, 1H), 2.15 (m, 2H), 1.85 (m, 4H). ^{13}CNMR (75 MHz, DMSO-d_6) δ: 163.30, 148.18, 147.29, 147.22, 143.88, 143.54, 143.39, 137.09, 136.44, 132.84, 126.44, 126.35, 123.67, 133.63, 117.49, 117.28, 111.12, 57.88, 56.12, 56.11, 51.89, 51.87, 38.27, 29.88, 29.87. MS (ESI) m/z: 448.2 $(M + H)^+$.

*4.2.2.3. N-(5-(1-(4-Nitrobenzyl)piperidin-4-yl)-2-ethoxyphenyl)benzamide (**17**).* M.p.: 212–215 °C. IR (KBr, cm^{-1}) ν_{max}: 2950, 1676, 1523, 1351, 697. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.64 (s, 1H), 8.51 (d, J = 2.1 Hz, 1H), 8.19 (d, J = 7.8 Hz, 2H), 7.89 (m, 2H), 7.53 (m, 5H), 6.94 (dd, J = 2.1 Hz, 8.4 Hz, 1H), 6.85 (d, J = 8.4 Hz, 1H), 4.14 (q, J = 6.9 Hz, 2H), 3.62 (s, 2H), 2.95 (d, J = 10.8 Hz, 2H), 2.55 (m, 1H), 2.15 (m, 2H), 1.85 (m, 4H), 1.48 (t, J = 6.9 Hz, 3H). ^{13}CNMR (100 MHz, $CDCl_3$) δ: 165.23, 149.08, 146.55, 135.52, 135.47, 135.23, 132.87, 131.88, 128.93, 128.92, 127.97, 126.95, 126.94, 124.29, 124.25, 120.99, 119.14, 114.37, 111.57, 64.63, 60.07, 53.56, 53.55, 40.03, 30.23, 30.22, 14.90. MS (ESI) m/z: 460.3 $(M + H)^+$.

*4.2.2.4. N-(5-(1-(4-Nitrobenzyl)piperidin-4-yl)-2-ethoxyphenyl)pyrazine-2-carboxamide (**21**).* M.p.: 236–238 °C. IR (KBr, cm^{-1}) ν_{max}: 3362, 2934, 1681, 1535, 1347, 1018, 688. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.35 (br, 1H), 9.50 (d, J = 1.2 Hz, 1H), 8.79 (d, J = 2.7 Hz, 1H), 8.61 (dd, J = 1.5, 2.7 Hz, 1H), 8.53 (d, J = 2.1 Hz, 1H), 8.19 (dd, J = 2.1, 6.9 Hz, 2H), 7.54 (m, 2H), 6.96 (d, J = 8.4 Hz, 1H), 6.87 (d, J = 8.4 Hz, 1H), 4.16 (q, J = 6.9 Hz, 2H), 3.63 (s, 2H), 3.00 (m, 2H), 2.53 (m, 1H), 2.17 (m, 2H), 1.86 (dd, J = 3.0, 8.1 Hz, 4H), 1.51 (t, J = 6.9 Hz, 3H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 160.18, 148.13, 146.21, 143.97, 143.53, 143.45, 132.90, 130.00, 126.71, 123.50, 123.49, 122.51, 122.50, 117.47, 117.46, 112.22, 112.20, 79.12, 64.39, 64.38, 52.00, 38.29, 29.80, 29.79, 14.61. MS (ESI) m/z: 461.2 $(M + H)^+$.

*4.2.2.5. N-(5-(1-(4-Nitrobenzyl)piperidin-4-yl)-2-isopropoxyphenyl) benzamide (**25**).* M.p.: 124–125 °C. IR (KBr, cm^{-1}) ν_{max}: 3421, 2975, 1670, 1523, 1328, 1109, 699. ^{1}HNMR (DMSO-d_6, 300 MHz): δ 10.95 (br, 1H), 9.26 (s, 1H), 8.34 (d, J = 8.7 Hz, 2H), 7.95 (d, J = 8.7 Hz, 2H), 7.91 (d, J = 7.8 Hz, 2H), 7.58 (m, 3H), 7.06 (d, J = 8.4 Hz, 1H), 6.99 (br of d, J = 8.4 Hz, 1H), 4.60 (q, J = 6.3 Hz, 1H), 4.49 (d, J = 3.9 Hz, 2H), 3.45 (m, 2H), 3.10 (m, 2H), 2.77 (m, 1H), 1.98 (m, 4H), 1.30 (d, J = 6.3 Hz, 6H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 164.71, 148.09, 147.65, 137.23, 136.18, 134.60, 132.92, 132.43, 131.63, 128.63, 128.62,

128.61, 127.17, 127.15, 123.61, 123.06, 123.05, 121.60, 114.28, 71.64, 57.73, 51.78, 51.77, 38.18, 29.72, 29.70, 21.88, 21.86. MS (ESI) *m*/*z*: 474.3 $(M + H)^+$.

*4.2.2.6. N-(5-(1-(4-Nitrobenzyl)piperidin-4-yl)-2-isopropoxyphenyl) pyrazine-2-carboxamide (**29**).* M.p.: 215–218 °C. IR (KBr, cm^{-1}) ν_{max}: 3378, 2981, 1685, 1539, 1344, 1117, 856. ^{1}HNMR (DMSO-d_6, 300 MHz): δ 10.68 (br, 1H), 9.33 (d, J = 1.5 Hz, 1H), 8.97 (dd, J = 1.5, 2.4 Hz, 1H), 8.84 (dd, J = 1.5, 2.4 Hz, 1H), 8.41 (s, 1H), 8. 36 (d, J = 7.5 Hz, 2H), 7.94 (d, J = 7.5 Hz, 2H), 7.13 (d, J = 7.8 Hz, 1H), 6.98 (br of d, J = 7.8 Hz, 1H), 4.68 (q, J = 5.4 Hz, 1H), 4.51 (d, J = 4.8 Hz, 2H), 3.47 (m, 2H), 3.15 (m, 2H), 2.80 (m, 1H), 2.03 (m, 4H), 1.36 (m, 6H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 160.15, 148.16, 148.10, 145.29, 143.95, 143.53, 143.50, 137.20, 136.78, 132.92, 132.90, 127.79, 123.63, 123.31, 122.41, 117.45, 114.31, 71.69, 57.75, 51.78, 51.77, 38.37, 29.73, 29.71, 21.92, 21.90. MS (ESI) *m*/*z*: 476.2 $(M + H)^+$.

4.2.3. Procedure C

*4.2.3.1. N-((5-(1-(4-Aminobenzyl)piperidin-4-yl)-2-methoxy)phenyl) benzamide (**10**).* A solution of compound **9** (70 mg, 0.16 mmol) in methanol (50 mL) was hydrogenated in the presence of 5% Pd/C (7 mg) under a H_2 atmosphere (57 psi) at room temperature for 24 h. The mixture was filtered, and the filtrate was concentrated to give foam (65 mg, 99% yield). M.p.: 210–211 °C. IR (KBr, cm^{-1}) ν_{max}: 3418, 2839, 1655, 1024, 712. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.54 (s, 1H), 8.47 (s, 1H), 7.89 (d, J = 7.8 Hz, 2H), 7.52 (m, 3H), 7.13 (d, J = 7.5 Hz, 2H), 6.95 (d, J = 8.4 Hz, 1H), 6.85 (d, J = 7.5 Hz, 1H), 6.66 (d, J = 6.9 Hz, 2H), 3.90 (s, 3H), 3.62 (s, 2H), 3.44 (s, 2H), 3.00 (d, J = 10.3 Hz, 2H), 2.49 (m, 1H), 2.02 (m, 2H), 1.82 (m, 4H). ^{13}CNMR (75 MHz, DMSO-d_6) δ: 164.63, 147.92, 135.60, 134.47, 132.50, 132.46, 132.45, 131.26, 128.56, 128.55, 128.29, 127.80, 127.14, 126.80, 126.80, 124.91, 122.01, 119.41, 110.37, 55.66, 55.55, 51.73, 51.70, 38.71, 29.86, 29.00. MS (ESI) *m*/*z*: 416.3 $(M + H)^+$.

Procedure C was applied to prepare compounds **14**, **18**, **22**, **26**, **30**.

*4.2.3.2. N-(5-(1-(4-Aminobenzyl)piperidin-4-yl)-2-methoxyphenyl) pyrazine-2-carboxamide (**14**).* M.p.: 209–211 °C. IR (KBr, cm^{-1}) ν_{max}: 3306, 2836, 1672, 1541, 1020, 808. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.25 (br, 1H), 9.49 (d, J = 1.5 Hz, 1H), 8.78 (d, J = 2.7 Hz, 1H), 8.62 (dd, J = 1.5, 2.4 Hz, 1H), 8.51 (d, J = 2.1 Hz, 1H), 7.14 (d, J = 8.4 Hz, 2H), 6.98 (dd, J = 2.4, 8.7 Hz, 1H), 6.87 (d, J = 8.4 Hz,1H), 6.67 (d, J = 8.1 Hz, 2H), 3.95 (s, 3H), 3.45 (s, 2H), 3.02 (m, 2H), 2.51 (m, 1H), 2.04 (m, 2H), 1.82 (m, 4H). ^{13}CNMR (75 MHz, CD_3OD) δ: 165.00, 149.60, 148.91, 144.83, 142.50, 137.40, 134.54, 134.53, 134.40, 131.23, 128.15, 124.85, 124.84, 124.12, 119.40, 112.06, 112.05, 60.93, 56.79, 54.24, 54.23, 40.43, 31.83, 31.82. MS (ESI) *m*/*z*: 418.3 $(M + H)^+$.

*4.2.3.3. N-((5-(1-(4-Aminobenzyl)piperidin-4-yl)-2-ethoxy)phenyl) benzamide (**18**).* M.p.: 165–167 °C. IR (KBr, cm^{-1}) ν_{max}: 3417, 2926, 1688, 1535, 1141, 711. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.61 (s, 1H), 8.47 (d, J = 2.1 Hz, 1H), 7.88 (d, J = 6.9 Hz, 2H), 7.53 (m, 3H), 7.13 (d, J = 8.1 Hz, 2 Hz, 2H), 3.61 (s, 2H), 3.44 (s, 2H), 3.00 (d, J = 11.1 Hz, 2H), 2.48 (m, 1H), 2.02 (m, 2H), 1.83 (m, 4H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 164.81, 149.00, 136.09, 134.56, 132.75, 132.74, 131.61, 128.56, 128.55, 128.50, 127.26, 127.25, 127.21, 127.09, 123.27, 121.94, 121.68, 121.67, 112.54, 64.08, 58.35, 51.45, 51.40, 38.22, 29.75, 29.74, 14.62. MS (ESI) *m*/*z*: 430.3 $(M + H)^+$.

*4.2.3.4. N-(5-(1-(4-Aminobenzyl)piperidin-4-yl)-2-ethoxyphenyl) pyrazine-2-carboxamide (**22**).* M.p.: 180–182 °C. IR (KBr, cm^{-1}) ν_{max}: 3421, 2930, 1678, 1401, 1251, 809. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 10.33 (br, 1H), 9.49 (d, J = 1.5 Hz, 1H), 8.78 (d, J = 2.4 Hz, 1H), 8.61 (dd, J = 1.5, 2.4 Hz, 1H), 8.50 (d, J = 1.8 Hz, 1H), 7.14 (d, J = 9.0 Hz, 2H), 6.95 (dd, J = 2.4, 8.4 Hz, 1H), 6.86 (d, J = 8.4 Hz, 1H), 6.66 (d, J = 9.0 Hz, 2H), 4.14 (q, J = 6.9 Hz, 2H), 3.63 (br, 1H), 3.46 (s, 2H), 3.02 (d, J = 10.8 Hz, 2H), 2.50 (m, 1H), 2.07 (m, 2H), 1.82 (m, 4H), 1.50 (t, J = 6.9 Hz, 3H). ^{13}CNMR (100 MHz, CD_3OD) δ: 165.00, 148.68, 144.90, 137.35, 136.30, 134.55, 134.54, 134.12, 131.48, 128.38, 125.90, 125.00, 125.00, 124.05, 121.80, 119.15, 113.09, 65.89, 60.82, 54.17, 54.16, 40.44, 31.85, 31.84, 15.13. MS (ESI) *m*/*z*: 432.3 $(M + H)^+$.

*4.2.3.5. N-((5-(1-(4-Aminobenzyl)piperidin-4-yl)-2-isopropoxy) phenyl)benzamide (**26**).* M.p.: 165–167 °C. IR (KBr, cm^{-1}) ν_{max}: 3418, 2929, 1662, 1473, 1109, 710. ^{1}HNMR (DMSO-d_6, 300 MHz): δ 10.63 (br, 1H), 9.26 (s, 1H), 7.91 (d, J = 6.9 Hz, 2H), 7.82 (s, 1H), 7.57 (m, 5H), 7.20 (d, J = 7.5, 2H), 7.06 (d, J = 8.7 Hz, 1H), 6.98 (br of d, J = 7.2 Hz, 1H), 4.58 (q, J = 5.4 Hz, 1H), 4.26 (s, 2H), 3.39 (br of d, J = 10.8 Hz, 2H), 3.03 (m, 2H), 2.75 (m, 1H), 2.00 (m, 4H), 1.28 (d, J = 6.0 Hz, 6H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 164.71, 147.70, 147.69, 136.25, 134.80, 134.60, 132.78, 132.76, 131.63, 128.63, 128.60, 128.05, 127.17, 127.16, 123.11, 122.49, 122.48, 121.69, 114.30, 71.04, 58.25, 51.47, 51.46, 38.26, 29.71, 29.70, 21.89, 21.88. MS (ESI) *m*/*z*: 444.3 $(M + H)^+$.

*4.2.3.6. N-(5-(1-(4-Aminobenzyl)piperidin-4-yl)-2-isopropoxyphenyl)pyrazine-2-carboxamide (**30**).* M.p.: 168–169 °C. IR (KBr, cm^{-1}) ν_{max}: 3355, 2929, 1685, 1541, 1110, 821. ^{1}HNMR (DMSO-d_6, 300 MHz): δ 10.65 (s, 1H), 10.30 (s, 1H), 9.33 (s, 1H), 8.98 (s, 1H), 8.83 (s, 1H), 8.40 (s, 1H), 7.62 (d, J = 7.8 Hz, 2H), 7.56 (m, 3H), 7.38 (d, J = 7.8 Hz, 2H), 7.13 (d, J = 8.4 Hz, 1H), 6.97 (d, J = 8.4 Hz, 1H), 4.68 (q, J = 6.0 Hz, 1H), 4.30 (s, 2H), 3.42 (m, 2H), 3.20 (m, 2H), 2.90 (m, 1H), 2.04 (m, 4H), 1.13 (d, J = 6.0 Hz, 6H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 164.14, 148.16, 145.29, 143.96, 143.52, 143.50, 136.84, 135.10, 132.79, 132.78, 132.70, 128.00, 127.78, 122.44, 122.00, 117.45, 114.32, 71.70, 64.86, 58.31, 51.47, 38.47, 29.73, 29.70, 21.92, 21.90. MS (ESI) *m*/*z*: 446.3$(M + H)^+$.

4.2.4. Procedure D

*4.2.4.1. N-(5-(1-(4-Acetamidobenzyl)piperidin-4-yl)-2-methoxyphenyl)benzamide (**11**).* To a solution of compound **10** (300 mg, 0.67 mmol) in dichloromethane (30 mL) and pyridine (116 mg, 2.35 mmol), acetic anhydride (102 mg, 1.01 mmol) was added dropwise. After stirred at room temperature overnight, the resulting suspension was washed with brine, dried with $NaSO_4$. The CH_2Cl_2 solution was filtered, then the filtrate was concentrated. The residue was purified with chromatography (CH_2Cl_2/MeOH: 100/1) to give GL-2-49 190 mg in 62% yield. M.p.: 168–170 °C. IR (KBr, cm^{-1}) ν_{max}: 3415, 2938, 1670, 1519, 1536, 1024, 761. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.54 (s, 1H), 8.47 (s, 1H), 7.89 (d, J = 7.8 Hz, 2H), 7.54 (m, 5H), 7.30 (d, J = 8.4 Hz, 2H), 7.20 (br, 1H), 6.95 (dd, J = 1.8 Hz, 8.4 Hz, 1H), 6.85 (d, J = 8.4 Hz, 1H), 3.90 (s, 3H), 3.50 (s, 2H), 2.98 (d, J = 13.2 Hz, 2H), 2.51 (m, 1H), 2.17 (s, 3H), 2.07 (m, 2H), 1.84 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 168.00, 165.45, 147.63, 144.00, 140.00, 135.96, 135.26, 132.27, 131.98, 128.90, 128.89, 127.77, 127.10, 127.09, 125.00, 124.10, 121.38, 120.37, 119.27, 110.61, 56.20, 53.20, 40.01, 30.37, 29.71, 24.50, 22.50, 14.90. MS (ESI) *m*/*z*: 458.2 $(M + H)^+$.

Procedure D was applied to prepare compounds **15**, **19**, **23**, **27**, **31**.

*4.2.4.2. N-(5-(1-(4-Acetamidobenzyl)piperidin-4-yl)-2-methoxyphenyl)pyrazine-2-carboxamide (**15**).* M.p.: 181–183 °C. IR (KBr, cm^{-1}) ν_{max}: 3421, 2950, 1670, 1654, 1540, 1261, 1021, 709. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.54 (s, 1H), 8.46 (d, J = 1.8 Hz, 1H), 7.89 (m, 2H), 7.50 (m, 4H), 7.35 (d, J = 8.7 Hz, 2H), 6.95 (dd, J = 2.4 Hz, 8.4 Hz, 1H), 6.85 (d, J = 8.7 Hz, 1H), 3.90 (s, 3H), 3.58 (s, 2H), 3.03 (d, J = 13.2 Hz, 2H), 2.54 (m, 1H), 2.18 (s, 3H), 2.18 (m, 2H), 1.86 (m, 4H). ^{13}CNMR (100 MHz, CD_3OD) δ: 151.10, 146.30, 134.27, 134.26, 133.19, 133.11, 129.90, 129.89, 128.40, 128.39, 124.70, 122.90, 121.88, 121.75, 121.46, 113.10, 112.30, 112.15, 61.19, 56.62, 54.02, 53.91, 40.37, 31.89, 31.88, 19.60. MS (ESI) *m*/*z*: 460.2 $(M + H)^+$.

*4.2.4.3. N-(5-(1-(4-Acetamidobenzyl)piperidin-4-yl)-2-ethoxyphenyl)benzamide (**19**).* M.p.: 231–234 °C. IR (KBr, cm^{-1}) ν_{max}: 3089, 1733, 1700, 1535, 1252, 708. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 8.62 (s, 1H), 8.47 (d, J = 2.1 Hz, 1H), 7.88 (m, 2H), 7.50 (m, 5H), 7.29 (d, J = 8.1 Hz, 2H), 6.92 (dd, J = 2.1 Hz, 8.1 Hz, 1H), 6.83 (d, J = 8.1 Hz, 1H), 4.12 (q, J = 6.6 Hz, 2H), 3.50 (s, 2H), 2.98 (d, J = 10.8 Hz, 2H), 2.50 (m, 1H), 2.17 (s, 3H), 2.07 (m, 2H), 1.81 (m, 4H), 1.47 (t, J = 7.2 Hz, 3H). ^{13}CNMR (100 MHz, $CDCl_3$) δ: 169.77, 165.22, 146.57, 140.88, 135.85, 135.30, 132.13, 131.86, 130.00, 128.93, 128.92, 127.93, 126.98, 126.97, 123.00, 121.25, 120.37, 120.36, 119.11, 111.55, 64.63, 61.05, 53.09, 40.03, 30.33, 30.32, 25.88, 24.66, 14.92. MS (ESI) m/z: 472.2$(M + H)^+$.

*4.2.4.4. N-(5-(1-(4-Acetamidobenzyl)piperidin-4-yl)-2-ethoxyphenyl)pyrazine-2-carboxamide (**23**).* M.p.: 195–198 °C. IR (KBr, cm^{-1}) ν_{max}: 3366, 2929, 1674, 1596, 1499, 1020, 671. ^{1}HNMR ($CDCl_3$, 300 MHz): δ 9.40 (s, 1H), 8.89 (s, 1H), 8.77 (s, 1H), 8.47 (s, 1H), 7.74 (d, J = 9.0 Hz, 2H), 7.54 (d, J = 9.0 Hz, 2H), 7.06 (m, 2H), 4.37 (s, 2H), 4.22 (q, J = 7.2 Hz, 2H), 3.64 (m, 2H), 3.35 (m, 2H), 3.20 (m, 2H), 2.90 (m, 2H), 2.54 (m, 2H), 2.17 (s, 3H), 1.52 (t, J = 7.2 Hz, 3H). ^{13}CNMR (100 MHz, CD_3OD) δ: 171.99, 162.31, 148.91, 148.61, 146.10, 144.82, 141.80, 139.50, 137.40, 133.10, 133.09, 128.32, 126.10, 125.49, 124.07, 121.41, 119.06, 113.06, 65.88, 61.48, 53.85, 53.84, 40.48, 31.87, 31.86, 23.93, 15.12. MS (ESI) m/z: 474.3 $(M + H)^+$.

*4.2.4.5. N-(5-(1-(4-Acetamidobenzyl)piperidin-4-yl)-2-isopropoxyphenyl)benzamide (**27**).* M.p.: 226–228 °C. IR (KBr, cm^{-1}) ν_{max}: 3335, 2977, 1684, 1660, 1538, 1251, 710. ^{1}HNMR (DMSO-d_6, 300 MHz): δ 10.16 (s, 1H), 9.24 (s, 1H), 7.90 (m, 3H), 7.68 (d, J = 8.4 Hz, 2H), 7.56 (m, 3H), 7.48 (d, J = 8.4 Hz, 2H), 7.06 (d, J = 9.0 Hz, 1H), 6.97 (d, J = 6.6 Hz, 1H), 4.59 (q, J = 6.0 Hz, 1H), 4.25 (d, J = 5.1 Hz, 2H), 3.40 (m, 2H), 3.03 (m, 2H), 2.73 (m, 1H), 1.95 (m, 4H), 1.28 (d, J = 6.0 Hz, 6H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 168.55, 164.70, 147.61, 140.35, 136.26, 134.61, 131.97, 131.96, 131.63, 128.63, 128.60, 128.08, 127.16, 127.15, 123.87, 123.08, 121.52, 118.83, 118.82, 114.26, 71.04, 58.65, 51.35, 51.34, 38.29, 29.79, 29.78, 23.99, 23.98, 21.88, 21.87. MS (ESI) m/z: 486.3 $(M + H)^+$.

*4.2.4.6. N-(5-(1-(4-Acetamidobenzyl)piperidin-4-yl)-2-isopropoxyphenyl)pyrazine-2-carboxamide (**31**).* M.p.: 162–164 °C. IR (KBr, cm^{-1}) ν_{max}: 3350, 2929, 1681, 1594, 1540, 1110, 702. ^{1}HNMR (DMSO-d_6, 300 MHz): δ 10.31 (s, 1H), 10.21 (s, 1H), 9.34 (s, 1H), 8.97 (s, 1H), 8.84 (s, 1H), 8.40 (s, 1H), 7.68 (d, J = 8.4 Hz, 2H), 7.52 (d, J = 8.4 Hz, 2H), 7.13 (d, J = 8.4 Hz, 1H), 6.97 (d, J = 9.0 Hz, 1H), 4.70 (q, J = 5.7 Hz, 1H), 4.25 (s, 2H), 3.43 (m, 2H), 3.03 (m, 2H), 2.78 (m, 1H), 2.08 (s, 3H), 1.99 (m, 4H), 1.35 (d, J = 5.7 Hz, 6H). ^{13}CNMR (100 MHz, DMSO-d_6) δ: 168.56, 160.15, 148.16, 145.21, 143.97, 143.52, 143.51, 136.87, 131.97, 131.96, 127.80, 127.79, 123.86, 122.46, 118.83, 118.82, 117.41, 114.31, 71.70, 64.86, 58.67, 51.33, 51.32, 38.49, 29.79, 29.78, 23.99, 21.93. MS (ESI) m/z: 488.3 $(M + H)^+$.

4.3. Molecular modeling protocols

All ligands used in the docking studies were built with standard bond lengths and angles using the molecular modeling package SYBYL-X 2.0. The small molecules were assigned Gasteiger–Huckel charges and energy minimized with the Tripos Force Field.

All molecular modeling was collected using the SYBYL-X 2.0 molecular modeling package (Tripos LP, St. Louis, MO) on dual-core AMD Opteron(tm) 2.4 GHz processors. The amino acid sequence of chemokine receptor CCR5 was obtained from UniProtKB/Swiss-Prot (P51681). Within ClustalX a multiple alignment was performed with a gap opening penalty of 15 using the BLOSUM protein weight matrix series. Sequence alignment between CCR5 and CXCR4 was further optimized based on the most conserved residues among most GPCRs and used for model construction for both the inactive and active models. The comparative modeling software, MODELLER 9v8, was used to generate 100 homology models for each state using the default parameters.

Model screening was performed by using the genetic-algorithm docking program GOLD 5.1 (Cambridge Crystallographic Data Centre, Cambridge, UK) to dock maraviroc into the CCR5 homology models using GOLD score as the fitness function. Once receptor model was chosen based upon the discrete optimized protein energy (DOPE) scores, fitness function values, and the electronic and steric interactions between the ligands and receptor, further model refinement was done by using molecular mechanics based energy minimization in Sybyl-X 2.0. Briefly, the model was minimized using a Tripos Force Field with Gasteiger–Hückel charges, a non-bonded interaction cutoff of 8 Å with a distance-dependent dielectric constant of $\varepsilon = 4$ being terminated at 0.05 kcal/(mol Å). The minimized models were then analyzed using PROCHECK and ProTable within SYBYL-X 2.0 to ensure the overall quality of the models (i.e. acceptable torsion angles, steric clashes, bond lengths, etc.).

Four CCR5 antagonists (including maraviroc) were docked into the CCR5 homology model using GOLD 4.1. The compounds were docked twenty times each with standard GOLD parameters and the default GOLD scoring was used. For all the ligands the best GOLD-docked solutions were selected and merged into the CCR5-receptor. The combined receptor–ligand structures were energy-minimized following the method described above. The minimization step optimized the interaction between ligands and the CCR5 homology model by removing clashes and lowering strain energy.

4.4. Biological screening protocols

4.4.1. CCR5 calcium inhibition assays

CCR5-MOLT-4 cells (Obtained through the AIDS Research and Reference Reagent Program, NIAID, NIH, from Dr. Masanori Baba, Dr. Hiroshi Miyake, Dr. Yuji Iizawa) were transfected with Gqi5 pcDNA1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's recommended procedure and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 u/mL penicillin, 100 μg/mL streptomycin, and 1 mg/mL G418 at 37 °C and 5% CO_2. 48 h after transfection a total of 2,500,000 cells were spun down and brought back up in 8 mL of 50:1 HBSS:HEPES assay buffer. Cells were then plated at 25,000 cells per well into a clear bottom, black 96-well plate (Greiner Bio-one) and 50 μL of Fluo4 loading buffer (40 μL 2 μM Fluo4-AM (Invitrogen), 100 μL 2.5 mM probenacid, in 5 mL assay buffer) was added to bring the volume up to 130 μL. After incubating for 45 min, 50 μL of varying concentrations of ligands and controls were added and the plate was incubated for an additional 15 min. Plates were then read on a FlexStation3 microplate reader (Molecular Devices) at 494/516 ex/em for a total of 120 s. After 16 s of reading, 20 μL of 200 nM RANTES (Biosource) in assay buffer, or assay buffer alone, was added to the wells to bring the total volume up to 200 μL. The changes in Ca^{2+} mobilization were monitored and peak height values were obtained using SoftMaxPro software (Molecular Devices). Non-linear regression curves and IC_{50}'s were generated using GraphPad Prism. All experiments were repeated a total of three times.

4.4.2. Anti-proliferation assays

All cell lines, PC-3 and M12, were incubated at 37 °C in the presence of 5% CO_2. RPMI 1640 serum free media (GIBCO Invitrogen) containing 1% L-glutamine, 0.1% ITS (insulin, 5 μg/ml; transferrin, 5 μg/ml; and selenium, 5 μg/ml; Collaborative Research, Bedford) and 0.1% gentamicin was used to cultivate all cells. M12 cells were first incubated in media with 5% fetal bovine serum

(FBS); after 24 h serum free media was added with 0.01% epidermal growth factor (EGF). DU-145 and PC-3 cell lines were incubated in media containing 10% FBS at all times.

Prostate cancer tumor cells (PC-3, and M12) were plated into 96 well plates (BD Falcon, VWR) at a concentration of 1000 cells per well. Each cell line was plated in its respective serum containing media for a total concentration of 100 μL per well. After 24 h, various concentrations of drugs in a 50 μL PBS solution were added to the cells. Control cells were given 50 μL of PBS. Seventy-two hours after incubation with drug, the serum containing media was replaced with 100 μL of a 9:1 solution of serum free media and WST-1 (Roche). After 3 h of incubation with WST-1, the absorbance of each well was measured by a microplate reader (FlexStation3, Molecular Devices). Absorbances values were obtained using SoftMaxPro software (Molecular Devices) and non-linear regression curves were generated using Prism (GraphPad) to calculate IC_{50} values.

4.4.3. Basal cytotoxicity assay utilizing neutral red uptake (NRU)

NIH-3T3 cells were routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM, with high-glucose, L-glutamate, and sodium pyruvate; Invitrogen) supplemented with 10% new born calf serum (NBCS, Invitrogen) and 1% penicillin:streptomycin. Mouse embryonic fibroblast cells (NIH-3T3) were plated into 96 well plates (Costar, Corning) at a concentration of 2000 cells/well/100 μL. Plates were incubated at 37.5 °C, 5% CO_2 for 24 h. At that point, media was discarded from the plates and 50 μL of fresh culture media was added to the wells. Plates were then treated with 50 μL of compounds at various concentrations in a dilution media made up of DMEM with 1% penicillin:streptomycin. Control wells were given 50 μL of the dilution media. After 48 h of incubation, media was removed from the plates, each well was washed with 200 μL of Hank's Buffered Salt Solution (HBSS, with calcium and magnesium, Invitrogen) and the rinsing solution was removed from the plates. To each well, 200 μL of 25 μg/mL of neutral red (NR, 0.33% solution in DPBS; Sigma) in DMEM containing 5% NBCS and 1% penicillin:streptomycin was added and plates were incubated for 3 ± 0.1 h. After incubation, NR media was removed from the plates and each well was washed with 200 μL of HBSS. The washing solution was decanted from the plates and 100 μL of a solution containing 50% ethanol, 49% H_2O, and 1% glacial acetic acid was added. Plates were shaken rapidly for 20 min while being protected from light. Once removed from the shaker, plates were allowed to sit for 5 min and absorbance at 540 nM was measured by a microplate reader (FlexStation3, Molecular Devices). Absorbance values were obtained using SoftMaxPro software (Molecular Devices) and TC_{50} values were calculated using non-linear regression curves on Prism (GraphPad).

4.4.4. In vivo M12 tumor growth inhibition of the lead compound

Mice (each group of ten, either control group or drug group) were injected subcutaneously with 2×10^6 M12 cells. When tumors reached a measurable size of 33 mm^3, each mouse was injected in the tail vein with 0.3 mg/kg of compound **23** every four days over a sixteen days period. The controls were injected with saline. At the time of necropsy, tumor size was established by gross tumor mass and estimated tumor volume and analyzed.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejmech.2013.09.004.

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