Design, Synthesis and Biological Evaluation of Novel Piperazine Derivatives as CCR5 Antagonists

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Abstract

By using a fragment-assembly strategy and bioisosteric-replacement principle, a series of novel piperazine derivatives were designed, synthesized, and evaluated for their cellular target-effector fusion activities and in vitro antiviral activities against HIV-1. Preliminary structure-activity relationships (SARs) of target compounds were concluded in this study, and five compounds were found to exhibited medium to potent CCR5 fusion activities with IC50 values in low micromolar level. Among evaluated compounds, 23 h was found to be a CCR5 antagonist with an IC50 value of 6.29 µM and an anti-HIV-1 inhibitor with an IC50 value of 0.44 µM.

Introduction

AIDS, one of the leading threats for human health worldwide, is a disease of the human immune system caused by the human immunodeficiency virus (HIV). Although the highly active antiretroviral therapy (HAART) is an available option for AIDS treatment, many patients are suffered from incomplete efficacy, severe toxicity, and the eventual emergence of resistance [1]. Therefore, the development of potent antiretroviral agents with novel mechanism of action is of great interests in the field of medicinal chemistry and drug discovery.

C-C Chemokine receptor 5 (CCR5), a G protein-coupled receptor (GPCR) for the β-chemokines MIP-1α, MIP-1β, and RANTES [2] and a primary co-receptor with CD4 for macrophage-tropic (M-tropic or R5) HIV-1 viruses [3] has been identified as a new target for HIV-1 epidemic prevention and treatment. Efforts devoted to the development of CCR5 antagonists have resulted in the discovery of the first marketed small-molecule inhibitor against CCR5, maraviroc (UK-427,857, 1, Figure 1) [4]. Besides, several other promising molecules are currently under clinical trials as potential anti-HIV agents, such as the Takeda disclosed compound TAK-220 (2) [5,6].

Although reported CCR5 antagonists are of different structures, the presence of one basic nitrogen center that tends to form strong salt-bridge interaction with the Glu283 residue of CCR5 receptor was found to be one of the most important features for CCR5 antagonists. A hydrophobic interaction involving the Ile198 residue was found for both maraviroc and TAK-220, together with a T-shaped π-π stacking interaction involving the Trp86 residue (Figure 2) [7]. Thus, a ‘Y shape’ pharmacophore model that contains one basic center, three hydrophobic domains, and an amide linker (Figure 3) was proposed in this study.

Results and Discussion

Chemistry

Target compounds listed in Tables 1, 2, and 3 were synthesized as outlined in Figure 4, 5, and 6. As shown in Figure 4, benzaldehydes 3a–c were reacted with malonic acid and ammonium acetate to give β-amino acids 4a–c, which was reduced to γ-amino alcohols 5a–c with the presence of LiAlH4 [11]. Acylation of 5a–c with corresponding benzoyl chlorides afforded amidines 6a–d. Compounds 6a–d were then subjected to a Swern oxidation to give aldehydes 7a–d, whose following reductive amination with substituted phenylpiperazine hydrochlorides 8a–c [12] afforded target compounds 9a–h.

Based on the ‘Y shape’ pharmacophore model, a series of 1,3-diamine compounds that combined the structural features of maraviroc and TAK-220 (1 and 2) were designed by using a fragment-assembly strategy and bioisosteric-replacement principle. Hydrophobic aromatic rings with a variety of other functional groups were introduced to study structure-activity relationship of target compounds. Although some piperazine-based compounds have been reported before, the structures of compounds involved here are different from that of the reported papers [8–10]. Herein, we report the design, synthesis, and biological evaluation of the novel piperazine derivatives 9a–h, 13a–d, 17, and 23a–h, with a goal to discover novel compounds as potential CCR5 antagonists for HIV treatment.
Synthesis of 13a–d and 17 is depicted in Figure 5. Reaction of piperazine with 2-(4-chlorophenyl)acetonitrile afforded N-arylation compound 11a, which was submitted to reductive amination with aldehyde 7a in the presence of sodium triacetoxyborohydride to provide 13a. Compounds 11b–c were achieved by treatment with 4-cyanobenzoyl chloride/4-(chloromethyl)benzonitrile and piperazine, respectively. Compounds 13b–c were prepared from the corresponding intermediates 11b–c following the same procedure as described for the preparation of 13a from 11a. Target compound 13d was obtained by condensation mono-substituted piperazine 8c with β-amino acid 12, which was prepared by the acylation of β-phenylalanine 4a with 4-fluorobenzoyl chloride. Target compound 17 was obtained as follows. Reaction of aniline 14 with 1-bromo-3-chloropropane under microwave irradiation afforded compound 15. Acylation of 15 with 4-fluorobenzoyl chloride gave amide 16, whose reaction with 4-substituted piperazine 8c afforded target compound 17.

Target compounds 23a–h were synthesized through procedures as illustrated in Figure 6. Compound 18 was prepared by esterification of 4a, and then protected by BOC group to get ester 19, which was reduced to the required aldehyde 20 using DIBAL-H, and then reductive amination with the compound 8c furnished the key BOC-protected intermediate 21. A BOC-deprotection step gave precursor 22, whose acylation with corresponding carboxylic acids afforded target compounds 23a–h.

Biological Evaluation
A total of 21 novel piperazine derivatives (9a–h, 13a–d, 17, and 23a–h) were screened for their inhibitory activity against cell-cell fusion between target cells expressing CD4/CCR5 and effector cells expressing the envelope protein of HIV-1, gp-120 (fusion assay) [13]. All synthesized analogs, which were firstly tested in MTT assay, showed no significant cytotoxicity against HEK 295 cells at a concentration of 50 μM.

Among the first set of compounds with three hydrophobic phenyl rings and varied substituents on phenyl rings (Table 1), compound 9e exhibited the most potent CCR5 fusion activity with an IC_{50} value of 0.64 μM. The data in Table 1 clearly showed limited tolerance toward different substitutions (R_4) at β-position of hydrophobic aromatic ring, as only compounds with a cyano-substituent retained good activity (9e–9g, IC_{50}:0.64 to 10.01 μM). Replacement of the β-cyano group with methyl (9b), fluoro (9c), or amide (9h) resulted in a sharp loss of potency. Replacement of the 4-fluorophenyl ring (hydrophobic aromatic ring 1) of 9b and 9c with phenyl group (9a and 9d) did not significantly improve in vitro fusion potency. These results indicated that the presence of a cyano substituent at R^4 position is favorable for CCR5 fusion activity.

Thus, a further optimization process was initiated with the introduction of a chlorine atom at R^1 position or two chlorine atoms both at R^1 and R^2 positions of the phenyl ring 2 of 9e. Compound 9f with monochloro-substitution at R^1 position showed 5-fold less potent activity than that of 9e. 3,4-Dichloro-substituted analog (9g) demonstrated more than 15-fold less potent fusion activity in comparison to 9e. These results suggested that introduction of two hydrophobic function to the R^1 and R^2 positions is not favorable for fusion potency.
Figure 3. Proposed ‘Y shape’ pharmacophore model of CCR5 antagonists.
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Figure 4. Synthesis of target piperazine derivatives 9a–h.
Reagents and conditions: a) CH₃COONH₄, C₂H₅OH, reflux, 12 h; b) LiAlH₄, THF, 65 °C, 3 h; c) corresponding benzoyl chlorides, Et₃N, CH₂Cl₂, 0 °C, 4 h; d) (COCl)₂, DMSO, CH₂Cl₂, –78 °C, 2 h; e) NaBH(OAc)₃, Et₃N, CH₂Cl₂, rt, 8 h.

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We then turned our attention to the link between ring 2 and ring 3 of compound 9e, modifying the two basic nitrogen-atoms in piperazine and 4-cyanophenyl group. Replacing the cyano group at R3 with an acetonitrile group provided compound 13a, which was found inactive on CCR5-mediated fusion assay at a concentration of 10 \( \mu \text{M} \). Subsequent replacement of the 4-cyanophenyl group with 4-cyanobenzyl group afforded compound 13c, which was also inactive in fusion assay. A carbonyl group was introduced in the right (13b) or left (13d) side of the piperazine to reduce the basicity of the piperazine. Neither 13b nor 13d showed potent CCR5 fusion activity. Our observation suggested that 4-(piperazin-1-yl)benzonitrile is a favored scaffold for CCR5 antagonists. Interestingly, replacement of the phenyl ring 2 from the carbon atom to the nitrogen atom (17) resulted in a substantial loss in CCR5 fusion activity.

Modifications as shown in Table 2 were also made to explore SARs of target compounds. Replacement of the fluoro-substitution at phenyl ring in ring 1 with a hydrogen (23a, \( \text{IC}_{50} = 2.44 \mu \text{M} \)) or a chlorine atom (23b, \( \text{IC}_{50} = 2.87 \mu \text{M} \)) resulted in a 4- or 5-fold reduction in CCR5-mediated fusion activity. Conversion of the fluoro-substituent of 9e to a trifluoromethyl group (23c) led to a 15-fold loss in potency (\( \text{IC}_{50} = 10.00 \mu \text{M} \)). I-Isopropyl replacement (23d) was found to lead an even more sharp decrease in potency. These observations indicated that the electron withdrawing group at the 4-position in ring 1 rather than the corresponding electron-donating group contribute significantly in fusion activity. Not as expected, introducing of N-acetyl-piperidin-4-yl to R3 position (23e) resulted in a complete loss in fusion activity. So did benzyloxy-piperidine derivatives (23f). Compound 23g with a five-membered furan ring was found to be inactive in CCR5-mediated fusion assay. It was interesting that compound 23h containing an aliphatic six-membered ring instead of the aromatic ring was found to be well-tolerated (\( \text{IC}_{50} = 6.29 \mu \text{M} \)). As such, hydrogen- or halogen-substituted phenyl ring and cyclohexyl group were identified as the optimal substituents.

Fluorophenyl analogs 9e-g, phenyl, chlorophenyl, and trifluorophenyl analog 23a-c, and cyclohexyl analog 23h were evaluated for their anti-HIV activity using a recombinant HIV-1 virus pseudotyped with the envelope proteins of the CCR5-tropic virus (HIV-1 single cycle antiviral assay) [14]. For overall estimation of the therapeutic potential of these novel CCR5 antagonists, maraviroc was used as a positive control. Results are shown in Table 3. Albeit the observation of potent activity in fusion assay, compound 9e showed weak anti-HIV activity at a concentration of 10 \( \mu \text{M} \). Compound 23h showed potent anti-HIV activity in comparison with the other tested piperazine derivatives.

### Conclusions

A novel series of piperazine derivatives were synthesized by adopting a fragment-assembly strategy and the bioisosteric-replacement principle. Target compounds were evaluated for their CCR5-mediated fusion activity and cytotoxicity, which showed that five compounds displayed medium to potent CCR5 antagonist activity. Compound 23h was evaluated as a CCR5 antagonist with an \( \text{IC}_{50} \) value of 6.29 \( \mu \text{M} \) and an antiviral agent with an \( \text{IC}_{50} \) value of 0.44 \( \mu \text{M} \). The piperazine derivatives developed in this study, as well as the concluded SAR, might be useful for following optimization toward the development of novel CCR5 antagonists for HIV treatment.

### Table 1. Effects of different substituents R1-R4 and link between ring 2 and 3 on CCR5 fusion activity.

| Compd | R1 | R2 | R3 | R4 | inhibition rate (10 \( \mu \text{M} \)) a,b | \( \text{IC}_{50} \) (\( \mu \text{M} \)) a,b |
|-------|----|----|----|----|--------------------------------|----------------|
| 9a    | H  | H  | H  | CH3| 47%                          | –              |
| 9b    | H  | H  | F  | CH3| 35%                          | –              |
| 9c    | H  | H  | F  | F  | 41%                          | –              |
| 9d    | H  | H  | F  | F  | 26%                          | –              |
| 9e    | H  | H  | F  | CN| 20%                          | –              |
| 9f    | Cl | H  | F  | CN| 3.70                          | 10.01          |
| 9g    | Cl | Cl | F  | CN|                               |                |
| 9h    | H  | H  | F  | CONH2| 22%                          | –              |
| 13a   | inactive | – |          |
| 13b   | inactive | – |          |
| 13c   | inactive | – |          |
| 13d   | inactive | – |          |
| 17    | inactive | – |          |

*Mean value of at least two experiments.

### Table 2. Modification of the p-fluorophenyl moiety of 9e.

| Compd R5 | inhibition rate (10 \( \mu \text{M} \)) a,b | \( \text{IC}_{50} \) (\( \mu \text{M} \)) a,b |
|----------|--------------------------------|----------------|
| 23a      | phenyl-                          | 2.44          |
| 23b      | 4-chlorophenyl-                   | 2.87          |
| 23c      | 4-trifluoromethylphenyl-          | 10.00         |
| 23d      | 4-isopropylphenyl-               | 20%           |
| 23e      | 1-acetyl-piperidin-4-yl-          | inactive      |
| 23f      | 1-benzyl-piperidin-4-yl-          | inactive      |
| 23g      | furan-2-yl-                       | inactive      |
| 23h      | cyclohexyl-                       | 6.29          |

*Mean value of at least two experiments.

### Table 3. Antiviral activity of 9e-g, 23a-c, and 23h.

| Compd | inhibition rate (10 \( \mu \text{M} \)) a,b | \( \text{IC}_{50} \) (\( \mu \text{M} \)) a,b |
|--------|--------------------------------|----------------|
| 9e     | 23.8%                           | –              |
| 9f     | inactive                         | –              |
| 9g     | 2.9%                            | –              |
| 23a    | 28.9%                           | –              |
| 23b    | 29.9%                           | –              |
| 23c    | 24.6%                           | –              |
| 23h    | 66.5%                           | 0.44          |
| Maraviroc | 96.8%                          | 0.0011        |

*Mean value of two experiments.

aDMSO as a negative control.

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Materials and Methods

Cell Cytotoxicity Assays

Cytotoxicities of target compounds were tested by using MTT (Sigma-Aldrich) assay. Briefly, 100 μl of HEK293 cell suspension (5000 cells/well) were dispensed in a 96-well plate, and then preincubated the plate for 24 hours at 37°C in 5% CO2. 10 μl of various concentrations of substances to be tested were added to the plate. After 7 hours incubation, 10 μl of CCK-8 solution were added to each well of the plate. The plate was incubated at 37°C for another 2 hours in the incubator, the optical absorbance was measured at 430 nm using a microplate reader.

Two Stable Cell Lines

Effector cell line: This cell line express HIV envelope protein gp160 and chimera protein Rn-Dn. Rn-Dn is consist of the N terminal of renilla luciferase and the N terminal of DnaE intein from Anacystis nidulans R2 PCC7942.

Target cell line: This cell line express chemokine receptor 5(CCR5), CD4 protein and chimera protein Dc-Rc. Dc-Rc is consist of the C terminal of renilla luciferase and the C terminal of NnaE intein from Anacystis nidulans R2 PCC7942.

Cell-cell Fusion Assay (CCF Assay)

The cell-cell fusion assay was performed as described previously [13]. The effector cells were plated in 24 well white culture plates at 7.5×10^4 cell per well in DMEM supplemented with 10% FBS, 800 μg/mL G418. The target cells in the growth medium were then added to the plates at 7.5×10^4 cells/50 μL/well and incubated for 5 hours. At the end of coculture, 70 μL of renilla luciferase assay lysis was added into each well, and the cultures were gently shaken for 15 min. At the same time, add 20 μl of Renilla Luciferase Assay Reagent to the luminometer tube. Add

Figure 5. Synthesis of target piperazine derivatives 13a–d and 17. Reagents and conditions: a) 2-(4-chlorophenyl)acetonitrile, K2CO3, DMSO, reflux, 18 h; b) 4-cyanobenzyol chloride, Et3N, CH2Cl2, rt, 3 h; c) 4-(chloromethyl)benzoinitrile, THF, reflux, 2.5 h; d) NaBH(OAc)3, CH2Cl2, rt, 12 h; e) 4-fluorobenzyol chloride, Et3N, CH2Cl2, 0°C, 5 h; f) KI, K2CO3, CH3CN, reflux, 24 h.

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20 μl of cell lysate to the tube. Mix quickly by flicking the tube for 1 second. Place the tube in FB12 luminometer and initiate measurement. Luminescence was integrated over 1 second with a 2-second delay. When small molecule compounds needed to be added to the CCF assay system, the compounds were diluted manually in DMSO. Then, 10 μL of the diluted compounds was added to the effector cells just before the addition of target cells, thus making the final concentration of DMSO in the coculture 0.5%.

Plasmids
HIV-1 proviral indicator construct pNL-Luc-E- contains a full-length HIV-1 genome, in which env was replaced by firefly Luciferase coding sequence. pENV-Ad8 expresses R5-tropic envelope (AD8).

Viral Infectivity Assays
Single-cycle HIV-1 replication assays were performed as described previously [14]. In brief, 4×10⁵ 293T cells were cotransfected with 0.4 μg of pNL-Luc-E- and 0.4 μg of pENV-R5. After 48 h, the supernatant containing pseudovirion was harvested by filtration through a 0.45 μm filter and the amount of viral capsid protein was measured by p24 antigen capture ELISA (Biomerieux). The resultant supernatant (10 μL) was used to infect SupT1 cells (1×10⁵) in 96-well plates in the presence of testing compound at the concentration indicated. The SupT1 cells were lysed 48 h post-infection and firefly luciferase activities were determined using a firefly Luciferase Assay System (Promega). Values were normalized to the control group treated with DMSO, and represented relative infectivity of each sample testing.

Supporting Information
Experimental protocols, NMR data (¹H and ¹³C), Mass spectrometry data (MS and HRMS) and Melting points data of compounds are presented in File S1.

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File S1 Experimental protocols, NMR data (¹H and ¹³C), Mass spectrometry data (MS and HRMS) and Melting points data of compounds.

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Author Contributions
Conceived and designed the experiments: TL ZW YH NZ SC. Performed the experiments: ZW XD LC LM. Analyzed the data: TL ZW LC NZ SC. Contributed reagents/materials/analysis tools: XD LC NZ LM SC. Wrote the paper: TL ZW.

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