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Structure–activity relationships of novel, highly potent, selective, and orally active CCR1 antagonists

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Abstract—Design and synthesis of a series of 3-amino-4-(2-(2-(4-benzylpiperazin-1-yl)-2-oxoethoxy)phenylamino)cyclobutenedione derivatives as novel CCR1 antagonists are described. Structure–activity relationship studies led to the identification of compound **22**, which demonstrated potent binding activity, functional antagonism of CCR1 as well as good species cross-reactivity. In addition, compound **22** also showed desirable pharmacokinetic profiles and was selected for in vivo studies in the mouse collagen-induced arthritis model.

Chemokines are a specialized family of small cytokines (8–15 kDa) that act as potent mediators of inflammation by their ability to recruit and activate specific leukocyte subpopulations. Chemokines activate leukocytes by binding to specific chemokine receptors belonging to the G protein-coupled receptors (GPCR) on the target cell plasma membrane.[1,2]

Chemokines are divided into four subfamilies: CC, CXC, C, and CX_3C defined by the position of the conserved cysteine residues near the N-terminus. CCR1 is a chemokine receptor expressed by a variety of immune cells including monocyte-macrophages, T-lymphocytes, neutrophils, basophils, eosinophils, NK cells, mast cells, and dendritic cells. CCR1 is activated by CC chemokines such as MIP-1α, RANTES, and MPIF-1.[3] Substantial evidence regarding genetic and animal studies has linked CCR1 and its ligands to the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA) and multiple sclerosis, and to allograft rejection. CCR1-positive cells—mostly macrophages—are scattered throughout the synovium in RA.[4] MIP-1α null mice exhibited lower clinical and histopathological scores in anti-type II collagen monoclonal antibody (anti-CII mAb)-induced arthritis.[5] CCR1-deficient mice had reduced incidence and ameliorated severity of disease in a mouse EAE model.[6] CCR1-deficiency also significantly prolonged allograft survival in several cardiac allograft models.[7] The administration of Met-RANTES, a CCR1/5 antagonist, reduced joint inflammation, bone destruction, and cell recruitment into joints in rodent arthritis models.[8,9] Initial safety and proof-of-concept was shown with a selective, orally bioavailable CCR1 small molecule antagonist in patients with RA.[10] These data illustrate the potential of CCR1 antagonists in these diseases, and thus make it an attractive target for drug discovery research.

Several chemotypes of CCR1 antagonists have been reported: piperazine derivative I (BX-471)[11] and II,[12] xanthene derivative III,[13] hydroxyethylene peptide isostere compound **IV**[14], and piperidine derivative V[15] (Fig. 1). BX-471 and compound **IV** were advanced to the clinic for multiple sclerosis and rheumatoid arthritis, respectively.

Except compound **II**,[12a,2b] most CCR1 antagonists reported so far suffered from poor species cross-reactivity

Keywords: Chemokine receptor 1; CCR1 antagonist; Diaminocyclobutenedione.
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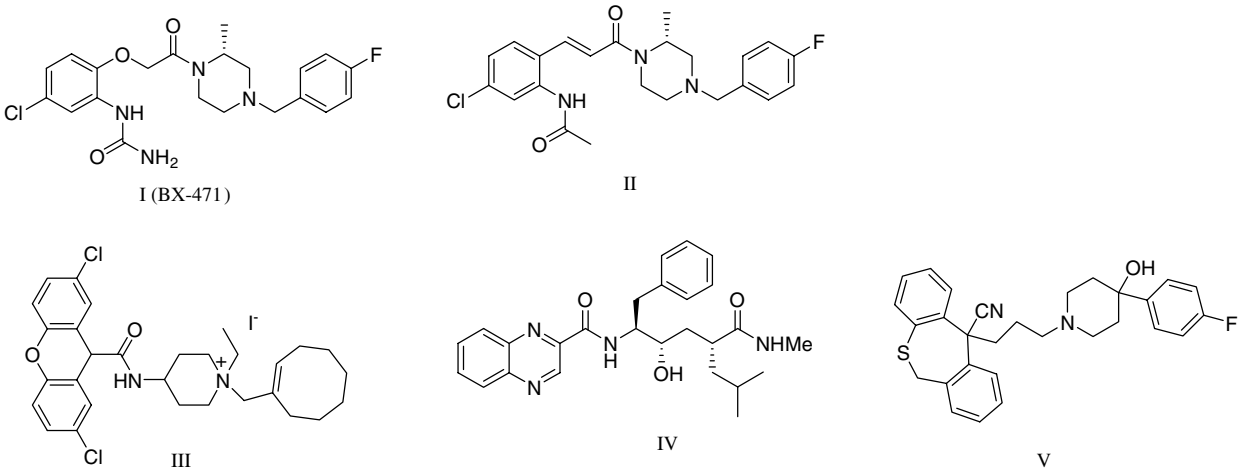


Figure 1. Selected CCR1 antagonists.

or poor oral bioavailability, which may limit extensive safety and pharmacological evaluation. For example, compound **III** has shown in vivo efficacy only by sc administration in the collagen-induced arthritis mouse model. Our objective was to design a novel series with an improved profile based on BX-471. A review of the

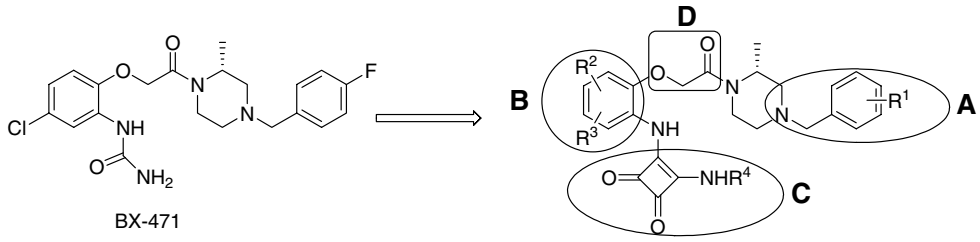
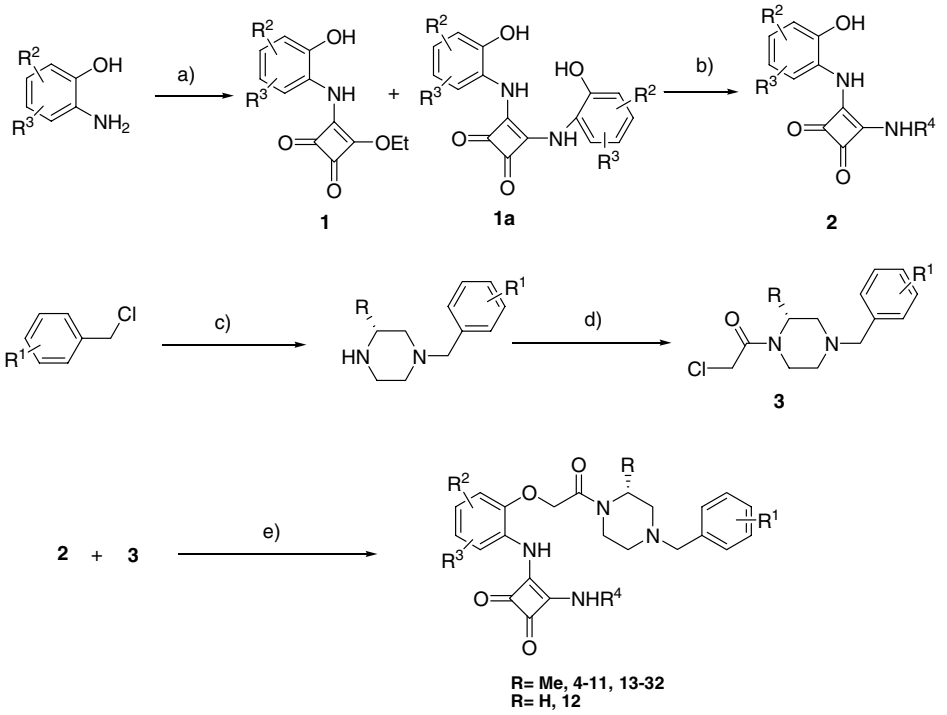
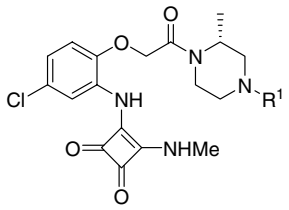


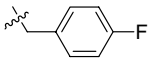
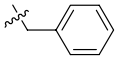
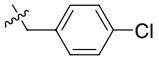
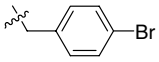
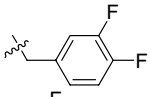
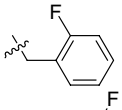
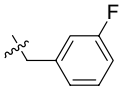
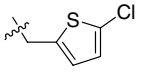
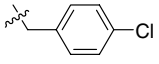
Figure 2. Cyclobutenedione as a urea bioisostere and SAR strategy.



Scheme 1. Reagents and conditions: (a) 1,2-diethoxycyclobutenedione, EtOH, reflux, 55–70%; (b) R^4NH_2, rt, 60–80%; (c) (R)-2-methylpiperazine or piperazine, DIEA, DCM, 75–90%; (d) chloroacetylchloride, DIEA, DCM, 70–85%; (e) K_2CO_3, KI, DMF, 65–75%.

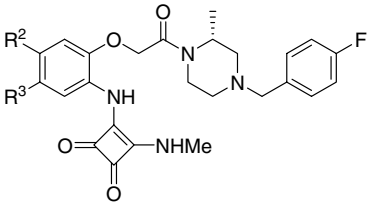
Table 1. Effect of substitutions on the *N*-benzylpiperazine moiety



Compound	R^1	IC_{50} (nM, hCCR1 binding)[a]
4		26
5		350
6		78
7		270
8		97
9		2,340
10		2,910
11		220
12	 (des-methyl piperazine)	200

[a] Results shown are mean values of triplicate samples in a single experiment.[16]

Table 2. Effect of substitutions on the left aryl ring



Compound	R^2	R^3	IC_{50} (nM, hCCR1 binding)[a]
4	H	Cl	26
13	H	H	920
14	H	Br	25
15	H	F	160
16	H	Me	90
17	F	F	140
18	H	Ph	>10,000

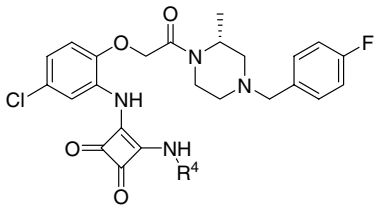
[a] Results shown are mean values of triplicate samples in a single experiment.[16]

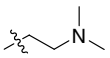
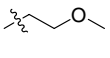
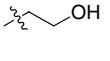
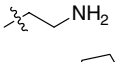
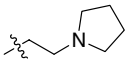
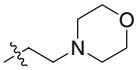
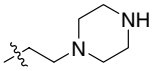
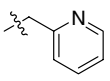
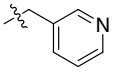
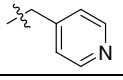
literature related to BX-471 indicated that the urea moiety is dispensable and can be replaced with various groups including hydrogen. We thus sought a urea replacement and identified diaminocyclobutenedione as a novel urea mimetic moiety. Here, we report the synthesis and structure–activity relationships (SAR) of a novel series of diaminocyclobutenedione derivatives as highly potent and selective CCR1 antagonists.

We separated the prototype structure into four discrete areas—benzyl piperazine (part A), left aryl (part B), aminocyclobutenedione (part C), linker (part D)—and conducted methodical SAR studies (Fig. 2).

Compounds **4**–**32** were synthesized according to the general Scheme 1, wherein the substitutions on the right and left aryls (R^1/R^2/R^3, parts A and B, Tables 1 and 2, compounds **4**–**18**) as well as the amine moiety on the

Table 3. Effect of amine substitutions on the cyclobutenedione moiety



Compound	R^4	IC_{50} (nM, hCCR1 binding)[a]
4	Me	26
19	Et	72
20	Pr	170
21	CH_2Ph	800
22	H	10
23		160
24		63
25		20
26		18
27		64
28		12
29		50
30		15
31		42
32		26

[a] Results shown are mean values of triplicate samples in a single experiment.[16]

cyclobutenedione (part C, Table 3, compounds **19**–**32**) were varied.

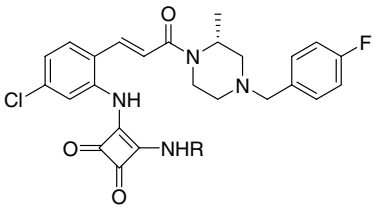
Requisite hydroxyanilines were reacted with 1,2-diethoxycyclobutenedione under EtOH reflux to give intermediate **1** and the corresponding diaminated compound **1a**. Treatment of **1** with desired amines at room temperature led to the diaminocyclobutenedione intermediate **2**, which was subsequently reacted with compound **3** (prepared in two steps) to yield target compounds **4**–**11** and **13**–**32**. The des-methyl piperazine compound **12** was prepared in a similar fashion starting from the 4-chloro-*N*-benzylpiperazine. The synthesis of compounds **33**–**34** wherein the linker $-OCH_2-$ was replaced with the bioisostere $-C{=}C-$ is outlined in Scheme 2.

The analogs prepared in this study were evaluated for their inhibitory activity against human CCR1 and expressed as IC_{50} values. Compound **4**, where a cyclobutenedione moiety was used to replace the urea functionality in BX-471, showed high potency for human CCR1 binding (IC_{50} = 26 nM). It was selected as the starting compound for systematic SAR studies. The effect of substitutions on the aromatic ring (part A, Table 1) was initially investigated. Unfortunately, all but 4-halo substitutions resulted in reduced potency. The potency decreased with the increase in size of the halogen (compare compounds **4**, **6**, and **7**). Compound **8**, the 3,4-difluoro analog, was 3-fold less potent than compound **4**. The un-substituted piperazine analog **12** also exhibited a 3-fold loss in potency.

The effect of substitutions on the phenyl ring (part B, Table 2) was explored next. Deletion of the 4-chlorine atom from **4** was detrimental to the potency, resulting in a >30-fold loss in activity (**13** vs **4**). The 4-fluoro analog (**15**) as well as 3,4-difluoro analog (**17**) led to a 6-fold loss in potency. Replacement of the 4-chloro with 4-bromo (**14**) retained the high potency with an IC_{50} value of 25 nM. On the other hand, replacement of the 4-chloro with the bulky phenyl group resulted in total loss of affinity, suggesting a space restriction around this site. Interestingly, replacement of the 4-chloro group of **4** with a methyl group retained modest potency (**16**, IC_{50} = 90 nM).

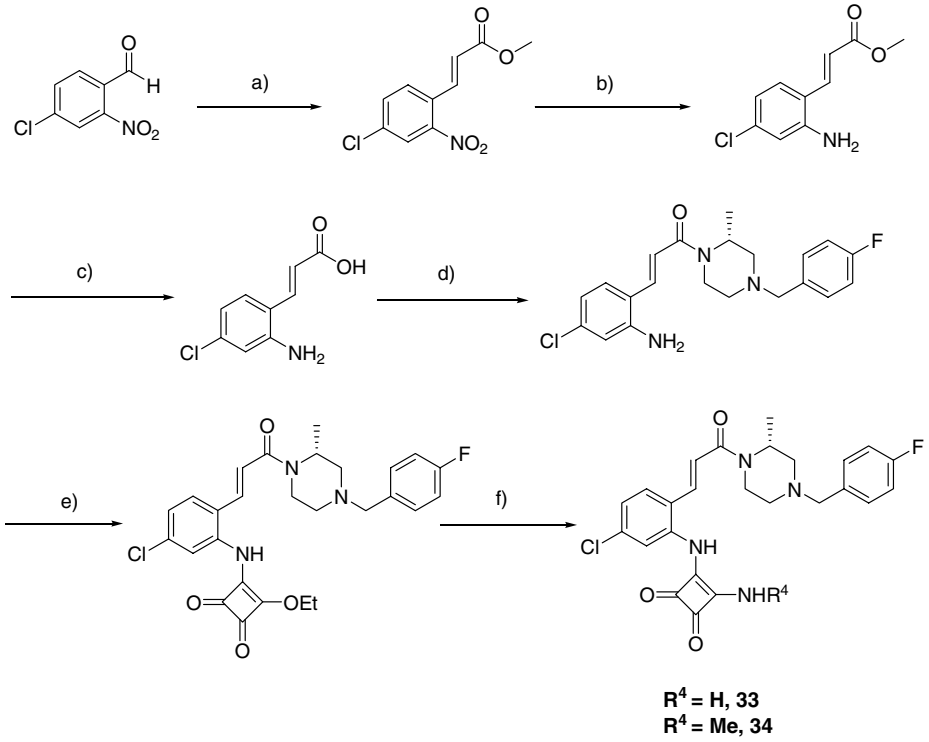
Having identified the 4-fluorobenzyl and 4-chloro-substituted phenyl as the two best moieties for parts A

Table 4. Linker modification



Compound	R	IC_{50} (nM, hCCR1 binding)[a]
33	H	7
34	Me	76

[a] Results shown are mean values of triplicate samples in a single experiment.[16]



R^4 = H, 33
R^4 = Me, 34

Scheme 2. Reagents and conditions: (a) $Ph_3P{=}CHCO_2CH_3$, toluene, reflux, 82%; (b) Zn, AcOH, EtOH, reflux, 30%; (c) 2 M NaOH, MeOH, 95%; (d) (R)-1-(4-fluorobenzyl)-3-methylpiperazine, EDC, HOBt, DIEA, THF, 68%; (e) 1,2-diethoxycyclobutenedione, EtOH, reflux, 42%; (f) R^4NH_2, EtOH, rt, 40% (R = H), 50% (R = Me).

Table 5. Functional and species activities of selected compounds

Compound	Ca^{2+} flux (h, IC_{50}, nM)	Chemotaxis (h, IC_{50}, nM)	mCCR1 binding (IC_{50}, nM)	rCCR1 binding (IC_{50}, nM)
4	18 ± 2	17	615	49
14	14 ± 5	25	—	—
22	4 ± 1	7	260	20
24	19 ± 1	54	—	—
25	13 ± 2	47	960	21
26	6 ± 1	23	968	17
28	12 ± 3	12	1770	87
29	5 ± 3	39	1670	24
31	8 ± 1	38	1000	130
33	6 ± 1	—	—	—
BX471	18 ± 2	18	615	59

Table 6. Pharmacokinetic profiles in rodents for compound **22**[a]

Species	C_{max} (μM, po)	T_{max} (h, po)	$AUC_{0\rightarrow\infty}$ (h*μM, po)	$t_{1/2}$ (h, po)	*F* (%)
Mouse	11.6	1	95.1	2.3	100
Rat	5.3	1	43.6	2.6	ND

ND, not determined.

[a] Male Balb/C mice and male Sprague–Dawley rats were used. Compound was administered as a solution in methyl cellulose with 10% Tween 80. AUC, area under the curve; *F*, bioavailability determined by iv/po ratio. Dose: 5 mg/kg iv; 50 mg/kg po.

and B, respectively, the focus was switched to modify the amino group on part C (Table 3). Increasing the size of the alkyl from Me (**4**) to Et (**19**) and Pr (**20**) gradually reduced the potency, whereas a 3-fold increase in potency was observed with the primary amine compound **22**. Incorporation of additional substitutions on the Et-chain of **19** either retained (**24**, **27**, and **29**) or enhanced potency (**25**, **26**, and **28**). The enhanced potency observed for the pyridyl analogs suggests that a hydrogen bond acceptor in this region of the molecule is favorable. SAR at this site also indicates the presence of a binding pocket that can accommodate diverse groups.

Table 4 highlights the modification of linker part D. Replacement of $–OCH_2–$ in **22** with the bioisostere –C≡C– in the linker part D produced the corresponding cinnamide analog **33** that retained similar potency (IC_{50} = 7 nM). However, compound **34**, the cinnamide analog of **4**, showed a 3-fold reduction in potency. Similar SAR with cinnamide analogs was observed previously.[12]

Selected compounds were examined for functional activity and species cross-reactivity[17] (Table 5). All compounds showed the inhibition of the MIP-1α-induced Ca^{2+} mobilization and inhibition of chemotaxis, which indicated functional antagonism for hCCR1. Compounds also showed higher affinity for rCCR1 than mCCR1. Compound **22** showed the best overall profile with IC_{50} values of 4 nM (Ca^{2+} mobilization), 7 nM (chemotaxis), 260 nM (mCCR1 binding), and 20 nM (rCCR1 binding), respectively.

Compound **22** was found to be a highly selective CCR1 antagonist with greater than 500-fold selectivity over CCR2, CCR3, CCR4, and CCR5 (data not shown). Based on its excellent in vitro potency, functional activity, species cross-reactivity, and pharmacokinetic profile (Table 6), compound **22** was selected for in vivo evaluation in a mouse collagen-induced arthritis model. It showed a statistically significant reduction of arthritis score at 50 mg/kg via oral administration. The details of these studies will be published in a separate paper.

In summary, we have identified a series of diaminocyclobutenedione analogs as novel, highly potent, selective, and orally active CCR1 receptor antagonists with functional activity and good species cross-reactivity against rat CCR1.

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16. Results shown are mean values of triplicate samples in a single experiment. THP-1 cells expressing CCR1 were harvested and washed once with PBS. Cells (5×10^6/ml) were mixed with test compound or vehicle and 50 pM human [^{125}I]MIP-1α in a 96-well tissue culture plate in a total volume of 120 μl of binding buffer which contained 50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 0.5% BSA (Hepes-BSA). The mixture was incubated at room temperature for 90 min and transferred to 96-well glass fiber filter plate pre-coated with 0.5% polyethyleneimine (PEI). Cells were separated by vacuum aspiration and washed twice with Hepes-BSA + 0.5 M NaCl. Radioactivity was measured on TopCount plate reader with 40 μl Microscint 40 scintillation fluid. Non-specific binding was determined in the presence of 150 nM unlabeled human MIP-1α.
17. Results shown for chemotaxis, mouse binding, and rat binding are the mean values of at least two independent experiments. *Ca^{2+} flux assay:* THP-1 cells in RPMI-1640 were adjusted to 2×10^6/ml, mixed 1:1 with Calcium Assay Kit buffer (Molecular Devices, Sunnyvale, CA), and incubated for 30 min at 37 °C in the presence of 2.5 mM probenicid (Sigma). Dye-loaded cells were then collected by centrifugation at 1000 rpm for 5 min, resuspended in assay buffer (RPMI-1640 containing 0.1% BSA), and seeded at 1.5×10^5/well on poly-D-lysine-coated 96-well plates (Molecular Devices) with or without test compounds. The plates were centrifuged at 1000 rpm for 5 min. After stimulation with 5 nM human MIP-1α, changes in intracellular free Ca^{2+} concentration were measured using FLIPR (Molecular Devices). *Chemotaxis assay:* THP-1 cells at 5×10^6/ml were incubated with 1 μM calcein (Molecular Devices) for 30 min at 37 °C. Cells were collected by centrifugation, washed once with RPMI-1640, and resuspended at 5×10^6/ml. The calcein-labeled cells were then mixed with compound or DMSO control for 30 min at 37 °C. and loaded in the wells on the top of a 5-μm polycarbonate filter in a 96-well Boyden chamber (NeuroProbe), in which 0.5 nM human MIP-1α with or without compounds had been added to the corresponding wells beneath the filter. The sealed chamber was incubated for 2 h at 37 °C. Non-migrated cells on top of the filter were removed by wiping. The filter was reversed and read at 485/530 nm emission/excitation wavelengths with FlexStation II (Molecular Devices).