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Discovery and Optimization of Pyrazole Amides as Antagonists of CCR1

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Key words: Chemokine Receptors; CCR1; Pyrazole; HTS hit; Development candidate

Abstract—A HTS screen for CCR1 antagonists afforded a novel sub-micromolar hit **5** containing a pyrazole core. In this report the design, optimization, and SAR of novel CCR1 antagonists based on a pyrazole core motif is presented. Optimization led to the advanced candidate compounds **(*S*)-16q** and **(*S*)-16r** with 250-fold improved CCR1 potency, excellent off-target selectivity and attractive drug-like properties.

The selective accumulation and activation of leukocytes in inflamed tissues contributes to the pathogenesis of inflammatory and autoimmune diseases. It has been suggested that chemokines and their receptors, which belong to a family of seven transmembrane G-protein coupled receptors (GPCRs), may be involved in this process. Chemokine Receptor 1 (CCR1) is a receptor for at least three chemokines (MIP-1α, MCP-3, and RANTES).[1-4] Despite set-backs in the use of CCR1 antagonists in autoimmune disease and inflammation, pharmacological intervention to disease via CCR1 remains of strong

interest. For example, recent publications discussed CCR1 antagonism for the potential treatment of systemic fungal infection or in oncology.[5,6]

A number of publications have disclosed ligands as antagonists of CCR1 and several have shown promising results in animal models of disease.[7-11] However, several early clinical candidates have failed to show efficacy (Figure 1):[12] BX-471 (**1**) failed to show efficacy in a Phase II clinical trial in patients with relapsing remitting multiple sclerosis (MS).[13-15] CP-481715 (**2**) failed to show efficacy in Rheumatoid Arthritis (RA) patients after 6 weeks of treatment. Compound **1** and **2** may not have achieved sufficient inhibition of the receptor since their PK profile led to high human doses. The Millennium compound MLN-3897 (**3**) was also stopped in the clinic after lack of efficacy in a Phase II clinical trial for RA.[16-18] It is presumed that compound **3** could not be dosed sufficiently high due to safety limitations. In 2012, ChemoCentryx reported positive proof of clinical concept for an oral CCR1 antagonist CCX354 (**4**; dose 200 mg, once daily) in a 12-week Phase II trial in patients (n = 160) with RA (ACR20 response at 12 weeks = 56%).[19-22] In contrast, in 2014, results from a 12-week Phase II trial in patients (n=123) with RA with BMS-817399 (**5**) did not show statistically significant differences in disease scores (ACR20, DAS28) or synovitis MRI read-outs despite achieving excellent coverage of the receptor.[23] Additional compound classes have been described and have advanced into clinical trials.[24,25]

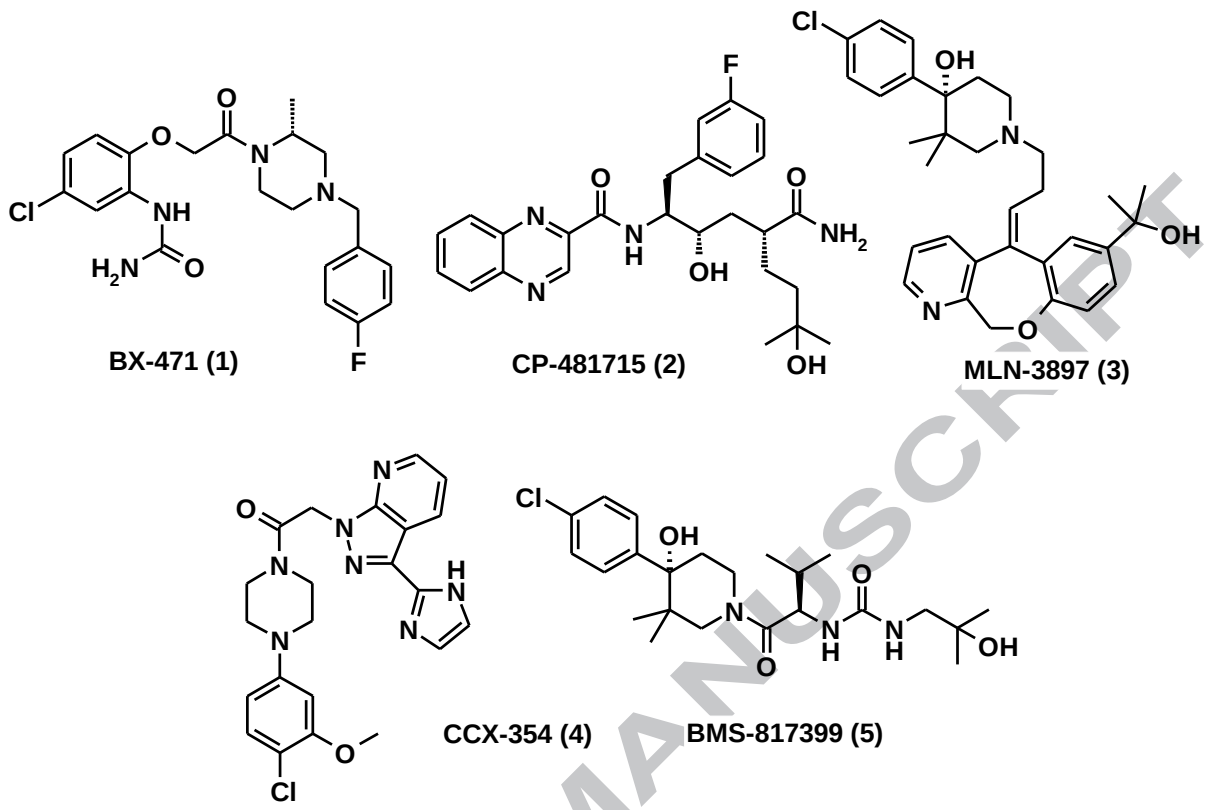


Figure 1: Clinical CCR1 antagonists

A CCR1 calcium flux assay high-throughput screening (HTS) campaign using the Boehringer Ingelheim compound collection was performed.[26] Several promising hit clusters were identified from this screen and subsequent ADME profiling yielded three compound classes of interest. Of these, the pyrazole class of compounds represented by **6** was prioritized as it was amenable to rapid structure-activity relationship (SAR) expansion via high-throughput synthesis (Figure 2). The compound showed sub-micromolar CCR1 potency (CCR1 calcium flux IC_{50} 500 nM), and showed some functional activity in a THP-1 chemotaxis assay (IC_{50} 1200 nM).[27] However, the compound was extremely lipophilic with a calculated logP of 5.8. This translated into very low aqueous solubility (<1 µg/mL).

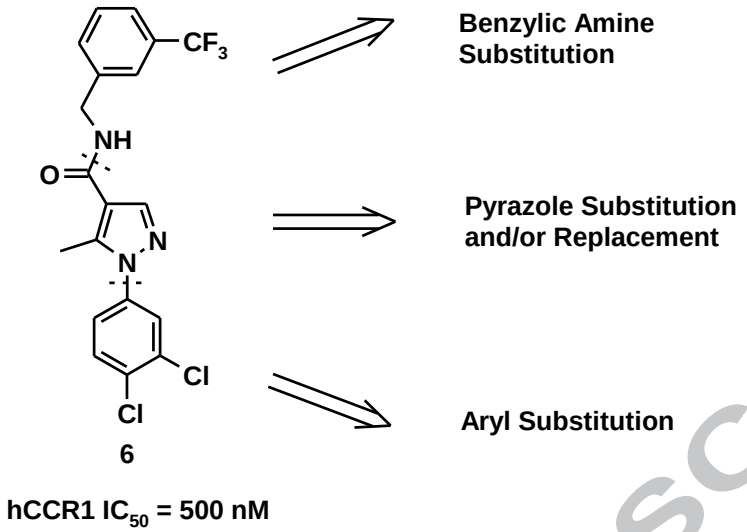
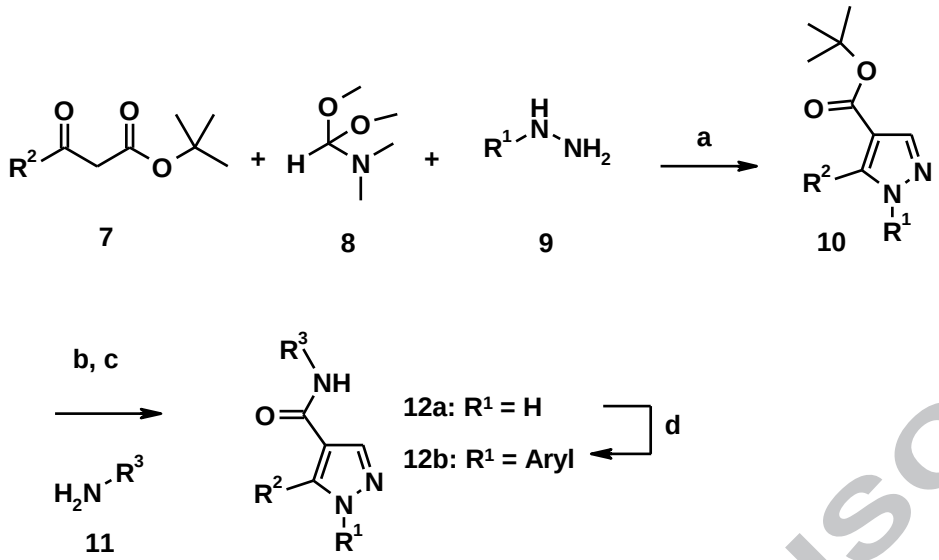


Figure 2. Pyrazole starting hit and SAR evaluation plan

The pyrazole portion of the molecules was generally prepared through cyclization of an alkyl β-oxoacetate **7** substituted with R^2 and dimethylformamide dimethylacetal (**8**) in the presence of acid (Scheme 1). The resulting intermediate was then reacted with a hydrazine salt **9** bearing R^1, in the presence of base to provide the pyrazole ester **10**. The ester was hydrolyzed to provide the corresponding carboxylic acid which is coupled with the desired benzylic amine **11** using standard coupling conditions to provide the desired amides **12**.

Alternatively the desired β-oxoacetate **7** substituted with R^2 was heated with dimethylformamide dimethylacetal (**8**), followed by the addition of hydrazine hydrate (**9**; R^1 = H) to provide the pyrazole ester **10** (R^1 = H). Conversion to the acid was then followed by coupling with the benzyl amine **11** as described above. Coupling of the resulting pyrazole **12a** (R^1 = H) with an iodoarene in the presence of CuI, base and a suitable diamine ligand, provided the desired amide **12b** (R^1 = aryl).

The synthesis of specific substituted benzyl amines **11**, and the post-coupling modification of some amide compounds **12** (e.g. to install sulfonamide substituents) has been described elsewhere.[28,29] Chiral amines were prepared using Ellman's sulfinamide chemistry from the corresponding bromopyridine.[30,31] The asymmetric synthesis of enantiopure chiral benzylamines has been described elsewhere.[32]

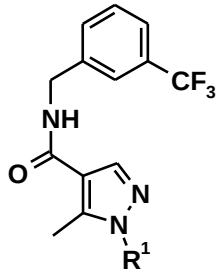


Scheme 1. Reagents and conditions: (a) TsOH, microwave; NEt_3, MeCN (b) HCl, dioxane (c) PyBop, Et_3N (d) substituted iodobenzene, K_2HPO_4, trans-*N,N'*-dimethylcyclohexane-1,2-diamine, toluene or DMF

Initial SAR efforts were focused on defining the minimum pharmacophore and improving the CCR1 potency of the screening hit **6** (Figure 2). Table 1 shows activity data for a number of *N*-1 substituted pyrazoles. The tolerance to variation of the *N*-1-substituent was extremely limited: A number of small alkyl groups for R^1 were prepared (data not shown) which were not active at the highest concentrations tested in the CCR1 calcium flux assay (CCR1 IC_{50} > 3000 nM). Removing the halogen atoms from the *N*-phenyl substituent such as in the unsubstituted phenyl analogue **13a,** led to a complete loss of CCR1 activity. Removing one halogen from **6** resulted in compounds with retained potency, such as in the *para*-fluorophenyl and *para*-chlorophenyl analogues **13b** and **13c** (CCR1 IC_{50} 790 and 600 nM, respectively). Mono-substitution in *meta*-position was not tolerated: The *meta*-chlorophenyl compound **13d** was inactive. *Ortho*-substitution was not tolerated either: The *ortho*-chlorophenyl analogue **13e** was also inactive. Compounds **13f-h** represent three examples of variation of the type of substituent: Neither a *meta*-tolyl group in **13f**, nor a *meta*-(trifluoromethyl)phenyl as in **13g** was tolerated, while the *para*-nitrophenyl compound **13h** retained some activity (CCR1 IC_{50} 960 nM). Replacing the phenyl group with a benzyl group as in **13i** was also not tolerated. No improvements in potency or physical chemical properties could be achieved through modification of the *N*-1 substitution. Since the original 3,4-

dichlorophenyl compounds such as **6** showed consistently very low aqueous solubility, the slightly less lipophilic but equally active *para*-chlorophenyl substituted compound **13c** was selected as a starting point for further SAR.

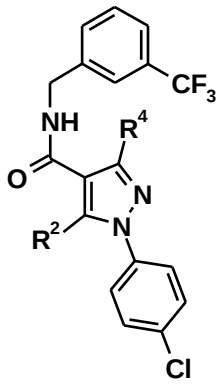
Table 1. *N*-1 substituted pyrazole SAR



Compound	R^1	CCR1 Ca^{2+} flux IC_{50} (nM)
6	3,4-dichlorophenyl	500
13a	phenyl	>3000
13b	4-fluorophenyl	790
13c	4-chlorophenyl	600
13d	3-chlorophenyl	>3000
13e	2-chlorophenyl	>3000
13f	3-methylphenyl	>3000
13g	3-(trifluoromethyl)phenyl	>3000
13h	4-nitrophenyl	960
13i	benzyl	>3000

Further optimization of compound **13c** began with an evaluation of pyrazole substitution and pyrazole replacements (Figure 2). Table 2 shows that the SAR in this moiety of the molecule was also extremely limited. Removal of the 5-methyl group as in **14a** resulted in loss of all activity. Replacement of the methyl group with trifluoromethyl as in **14b**, methoxymethyl as in **14d**, amino as in **14e,** or *N*,*N*-dimethylamino as in **14f** was not tolerated. Interestingly, the polar hydroxymethyl substituent in **14c** was tolerated (CCR1 IC_{50} 1200 nM), however this group introduced a metabolically labile benzylic oxidation site that resulted in significantly lower metabolic stability (human liver microsome (HLM) predicted clearance 80% of hepatic bloodflow ($\%Q_h$)). Introduction of an additional substituent in the 3-position in **14g** was not tolerated (CCR1 IC_{50} > 3000 nM).

Table 2. 3- and 5-substituted pyrazole SAR

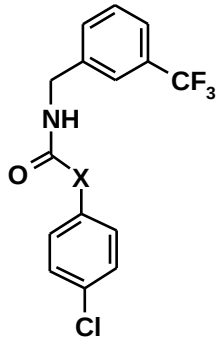


Compound	R^2	R^4	CCR1 Ca^{2+} flux IC_{50} (nM)
13c	Me	H	600
14a[a]	H	H	>3000
14b	CF_3	H	>3000
14c	CH_2OH	H	1200
14d	CH_2OMe	H	>3000
14e	NH_2	H	>3000
14f	$N(Me)_2$	H	>3000
14g[a]	Me	Me	>3000

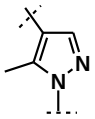
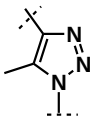
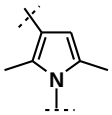
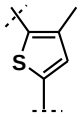
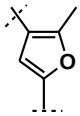
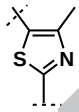
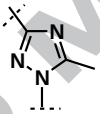
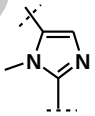
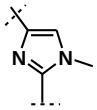
a) contains *N*-(3,4-dichlorophenyl) substituent on pyrazole

Next, the entire pyrazole ring was replaced with other scaffolds. The 1,2,3-triazole **15a** was 4-fold less active (CCR1 IC_{50} 2400 nM) than the pyrazole reference compound **13c**. Interestingly, the pyrrol **15b** was slightly more potent (CCR1 IC_{50} 350 nM) than the pyrazole **13c**. However, this moiety was not pursued further since it introduced potential reactive sites. None of the synthesized thiophene **15c**, oxazole **15d**, thiazole **15e**, 1,2,4-triazole **15f** and imidazole isomers **15g-h** retained any CCR1 activity.

Table 3. Pyrazole replacement SAR



Compound	X	CCR1 Ca^{2+} flux IC_{50} (nM)

13c		600
15a		2400
15b		350
15c		1900
15d		>3000
15e		>3000
15f		>3000
15g		>3000
15h		>3000

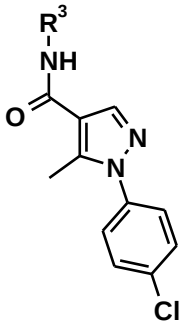
SAR exploration in the benzylamine moiety of the series was more fruitful (Figure 2). Table 4 shows selected examples. More than 500 compounds with various benzylamine moieties were explored, initially in directed libraries, and ultimately as single point analogs and combination molecules. Simple substitution with small and/or polar substituents on the benzylamine was not tolerated, such as in the 3-hydroxybenzyl compound **16a** or the 3-aminobenzyl compound **16b**. However, the high polar-surface area methylsulfonyl group was tolerated: The 3-methylsulfonyl compound **16c** retained activity with a modest loss of potency (CCR1 IC_{50} 2100 nM). While the same group was not tolerated in the 2-position in **16d**, the group could alternatively be placed in the 4-

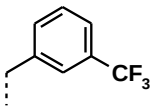
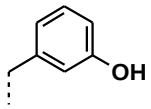
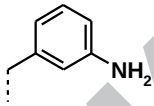
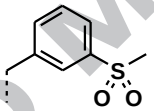
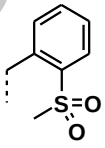
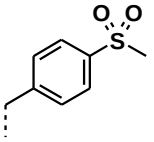
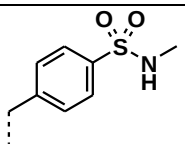
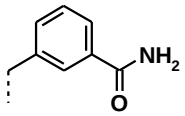
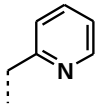
position as in **16e** (CCR1 IC_{50} 1700 nM). Related polar groups were explored and were generally not tolerated e.g. amide **16g** (CCR1 IC_{50} >3000 nM). Solely the sulfonamide **16f** retained activity (CCR1 IC_{50} 420 nM). Follow-up analogues revealed that the sulfonamide compounds in this series suffered from low solubility and the majority of SAR focused on the sulfones. Heteroaryl replacement to the benzyl group were not tolerated, all three pyridyl isomers **16h-j** were inactive. The combination of sulfonyl substitution and pyridyl replacement as in **16k** was equally inactive.

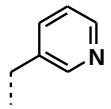
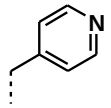
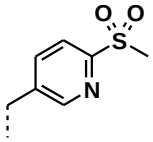
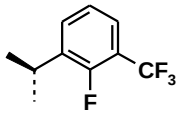
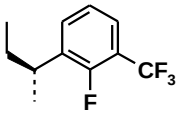
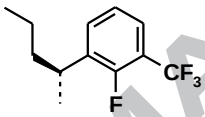
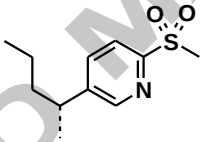
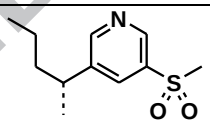
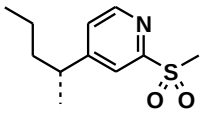
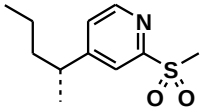
A break-through occurred when additional branching in the benzylic position was explored. The methylbenzyl analogue **16l** showed significantly improved potency (CCR1 IC_{50} 28 nM) that could be further improved by increasing the size of the branching substituent to ethyl in **16m** (CCR1 IC_{50} 13 nM) and *n*-propyl in **16n** (CCR1 IC_{50} 10 nM). These compounds were initially prepared using a slightly different substitution pattern on the benzyl ring (2-fluoro-3-(trifluoromethyl)phenyl) which increases potency by 2-3 fold over the 3-(trifluoromethyl)benzyl as in **13c**, however, the potency increase due to the added benzylic substituent was 10-20-fold. **16l-n** were prepared as single enantiomers from commercially available amines. It was later demonstrated that all activity resides in the (*S*)-isomer for chiral compounds in this series.[30]

In these compounds, polar modifications to improve physical chemical properties that were previously not tolerated, were not only tolerated, but led to further improvement in potency: The combination of heteroaryl replacement (pyridines), large polar substitution (alkylsulfonyl) and small alkyl substitution in the benzyl position (propyl) resulted in compounds with further increased potency and improved physical chemical properties: The pyridylsulfone **16o** showed acceptable potency (CCR1 IC_{50} 47 nM) without the use of overly lipophilic substituents (calculated logP 3.9). Moving the sulfonyl group to the preferred 3-position resulted in the identification of **16p** with a CCR1 IC_{50} of 15 nM. Additionally, moving the pyridyl nitrogen atom to the 4-position, resulted in the first single-digit nanomolar CCR1 antagonist from this series: **16q** showed a CCR1 IC_{50} of 3 nM. The *N*-methyl sulfonamide analogue **16r** was equipotent (CCR1 IC_{50} of 4 nM).

Table 4. Benzylamide SAR



Compound	R^3	CCR1 Ca^{2+} flux IC_{50} (nM)
13c		600
16a		>3000
16b		>3000
16c		2100
16d		>3000
16e		1700
16f		420
16g		>3000
16h		>3000

16i		>3000
16j		>3000
16k[a]		>3000
16l		28
16m		13
16n		10
16o		47
16p		15
16q		3.0
16r		4.0

a) contains *N*-(3,4-dichlorophenyl) substituent on pyrazole

16q and **16r** were initially synthesized in racemic form. The compounds were subsequently resolved into pure enantiomers and the active (*S*)-isomers were extensively profiled as potential development candidates.

Additional data for the two compounds **(*S*)-16q** and **(*S*)-16r** are shown in Table 5. The CCR1 potency of 2.0 and 2.9 nM IC_{50}, respectively, was approximately 250-fold more potent than the original screening hit **6**. This translated into functional activity in a THP-1

cell chemotaxis assay with 6.9 and 12 nM IC_{50}, respectively. Compounds of the series were minimally cross-reactive with mouse or rat CCR1 (mouse CCR1 binding IC_{50} >3000 nM and 600 nM, respectively) and, therefore, were not feasible to be tested in any *in vivo* PoP models. **(*S*)-16q** was tested for general receptor selectivity and showed no significant inhibition in a panel of 68 pharmacologically relevant receptors at the tested concentration of 10 μM, with the possible exception of 55% inhibition of melatonin MT_1 receptor at this concentration. The compounds **(*S*)-16q** and **(*S*)-16r** showed moderate inhibition of the hERG channel (22 and 9.6 M IC_{50}, respectively, in an IonWorks automated patch-clamp hERG study).

The drug-like properties of the **(*S*)-16q** and **(*S*)-16r** were attractive: The compounds had MW < 500 g/mole and were moderately polar with calculated logP values of 3.9 and 3.3, respectively. The simultaneous optimization of potency and properties had resulted in only a 19 and 38 g/mole increase in MW, while reducing the logP by over 2 units. The compounds showed good metabolic stability *in vitro* with <11% and 14% Q_h predicted clearance from HLM. No significant inhibition of Cytochrome P450 enzymes was detected. The compounds showed moderate solubility in a kinetic solubility screen at pH 7. No crystalline forms allowing determination of thermodynamic solubility were identified.

The compounds showed acceptable PK properties, for example **(*S*)-16q** showed low clearance of 10%Q_h, V_{SS} 1.3 L/kg, and excellent bioavailability of 87% in a rat PK study (Sprague-Dawley rat, po at 10 mg/kg in 0.5% methyl cellulose/0.015% tween 80 in water; iv at 1 mg/kg in 70% PEG400 in water).

Table 5. Profile of advanced compounds

Compound	CCR1 Ca^{2+} flux IC_{50} (nM)	CCR1 Chemotaxis IC_{50} (nM)[a]	HLM[b] CL (%Q_h)	CYP3A4 IC_{50} (μM)	HT Solubility pH 7 (μg/mL)	hERG IC_{50} (μM)[c]	MW	clogP
(*S*)-16q	2.9	6.9	<11	29	64	22	447	3.9
(*S*)-16r	2.0	12	14	>30	43	9.6	462	3.3

[a]CCR1 chemotaxis in THP-1 cells[27]
[b]Human liver microsomes (HLM)
[c]IonWorks Quattro automated patch clamp system

Compounds like **(*S*)-16q** and **(*S*)-16r** offered an attractive potent, selective CCR1 antagonist profile with excellent drug-like properties. The compounds were among the

best CCR1 antagonist available in the field with regard to their balance of physical-chemical properties and potency and selectivity. Ultimately, the compounds were not advanced into further pre-clinical testing (higher species PK, general safety pharmacology, exploratory toxicology studies), since at that time a series with significantly further improved potency was identified. The indazole series of CCR1 antagonists was designed through an innovative scaffold hopping approach using the pyrazole series as a starting point. The approach is described in a subsequent publication.

Competing Interest Statement: The authors have no competing interests to declare.

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(26) Compounds are assessed for the ability to block the interaction of CCR1 and its ligand in a functional cellular assay measuring calcium flux in response to MIP-1α in CCR1-transfected cells. Published CCR1 reference compounds gave the following IC_{50} values in this assay: BX-471 (**1**): 1.3 nM; CP-481715 (**2**): 59 nM; MLN-3897 (**3**): 0.8 nM; CCX354 (**4**): 2.0 nM; BMS-817399 (**5**): 3.0 nM.

Method A: Non-adherent cells purchased from Chemicon Corporation (HTS005C), stably expressing recombinant CCR1 and G-alpha-16 are grown in RPMI 1640 medium (Mediatech 10-080-CM) supplemented with 10% heat-inactivated FBS, 0.4 mg/mL Geneticin and penicillin/streptomycin. On the day of the assay, the cells are transferred to a beaker and dye-loaded in bulk using a Fluo-4 NW Calcium Assay Kit with probenecid (Invitrogen F36205) at 0.8E6 cells/mL for 1 hour at room temperature. After 1 hour, they are seeded in a 384-well tissue culture-treated plate at a density of 20,000 cells/well. Appropriately diluted test compound is added to the well to achieve a top concentration of 3,000 nM (diluted 3-fold with 10 doses total). The final concentration of DMSO is 1%. The buffer is HBSS (Invitrogen 14025) with 20 mM HEPES at pH 7.4. The cells are allowed to incubate 1 hour in the dark at room temperature. The plates are transferred to the FLIPR TETRA where MIP-1 alpha in 1% BSA is added at the EC80 final concentration. Wells +/- MIP-1 alpha containing diluted DMSO instead of compound serve as the controls. Intracellular calcium flux is recorded on the FLIPR TETRA, using excitation at 470/495 nm and emission at 515/575 nm. Data are analyzed using Activity Base software.

Method B: Non-adherent cells purchased from Chemicon Corporation (HTS005C), stably expressing recombinant CCR1 and G-alpha-16 are grown in RPMI 1640 medium (Mediatech 10-080-CM) supplemented with 10% FBS, 0.4 mg/mL Geneticin and penicillin/streptomycin. On the day of the assay, the cells are loaded with Calcium 4 dye

(Molecular Devices R7448) with Probenecid (Invitrogen P346400) at 8E5 cells/mL for 1 hour at room temperature. After 1 hour, they are seeded in a 384-well tissue culture-treated plate at a density of 20,000 cells/well. Appropriately diluted test compound is added to the well to achieve a top concentration of 3,000 nM (diluted 4-fold with 10 doses total). The final concentration of DMSO is 1%. The buffer is HBSS (Invitrogen 14025) with 20 mM HEPES at pH 7.4. The cells incubate 30 minutes at 37°C and then 30 minutes at room temperature. The plates are transferred to the HAMAMATSU FDSS6000 where MIP-1alpha in 1% BSA is added at the EC80 final concentration. All plates must be read within 4 hours of the start of dye-loading. Wells +/- MIP-1alpha containing diluted DMSO instead of compound serve as the controls. Data are analyzed using Activity Base software.

(27) Compounds are assessed for the ability to block the chemotaxis of THP-1 cells. BX-471 (**1**): 1.1 nM; CP-481715 (**2**): 39 nM; MLN-3897 (**3**): 1.8 nM; CCX354 (**4**): 4.0 nM; BMS-817399 (**5**): 1.5 nM.

THP-1 cells (500,000 cells/well, each plate will use 60 µL of cells per well at 1.1 x 10^7 cells/mL. Washed cells 3 times in assay buffer (HBSS + 10 mM HEPES pH 7.4 + 0.1% BSA (Sigma #A7030)) are added to media (Cell Gro #10-040-CM) add *L*-Glutamine (Cell-Gro # 25-005-CI (final 2 mM), Pen/strep (Cell-Gro # 30-002-CI) final 100 Units & FBS (Cell-Gro #35-015-CV) final 10%) in T175 flask and grown in 37˚C incubator with 5% CO_2. Cells grown between 2.0 x 10^5 and 1.0 x 10^6 cell/mL. Resuspended cells to 1.1 x 10^7 cells/mL. Compounds received as dry powder are dissolved in 100% DMSO (Dri-solve EMD #MX 1457-6) at 10 mM or as liquid in 15 µL tubes (10 mM). Added reagents by using a multidrop with a disposable head: 90 µL assay buffer to 96 well plate (Falcon 35-1190), 60 µL cell suspension to a 96 well plate (Falcon 35-1190), 280 µL of assay buffer to column 12 of chemotaxis chamber, 280 µL of 6.75 nM RANTES to columns 1-11 of chemotaxis chamber. Transferred 10 µL compound titration (100% DMSO) into 90 µL assay buffer mix 6 times. Transferred 6.7 µL diluted compound to 60 µL cells in Falcon 96 well plate. Cover using lids (Falcon 35-1191), mixed by shaking. Incubated cells for 30 minutes at 37°C and 5% CO_2. Changed tips and transferred 32 µL diluted compound to lower chamber containing 280 µL 6.75 nM RANTES (R&D systems #278-RN/CF). Placed membrane on lower chamber. After 30 min incubation, mixed cells on

shaker. On the Cybio added 50 µL cells to top of membrane. Incubated 3 hours at 37°C and 5% CO_2. In a 96 well (Falcon 35-1190) added 260 µL assay buffer to column 1 row A-D and 100 µL to columns 2-8 in Rows A-D. Added 50 µL cell suspension to column 1, rows A-D. Titrate 1:2 to column 7. Added lid (Falcon 35-1191) place in incubator for 3 hours. On Cybio mixed % chemotaxis plate and added 40 µL to white half area plate containing 40 µL Promega Cell Titer-Glo reagent (#G-7572). On cybio mixed lower chamber and added 40 µL to white half area plate containing 40 µL Promega Cell Titer-Glo reagent (#G-7572). Mixed on shaker. Incubated at room temperature for 15 min. Read luminescence on LJL Analyst. % Chemotaxis generated by excel template.

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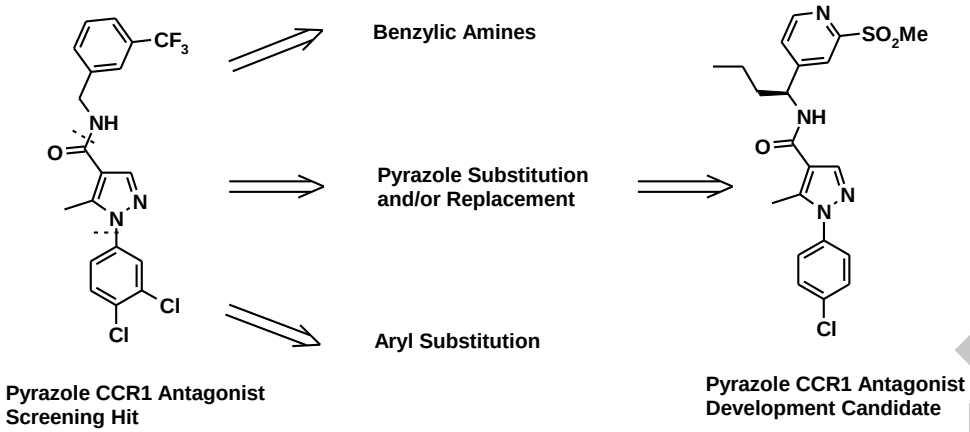
Highlights:

- A HTS screen for CCR1 antagonists afforded a novel hit containing a pyrazole core
- Multi parameter optimization led to advanced candidate compounds
- Candidates show 250-fold improved CCR1 potency compared to initial hit
- Candidates show excellent off-target selectivity, attractive drug-like properties

Graphical Abstract:



Pyrazole CCR1 Antagonist Screening Hit

Pyrazole CCR1 Antagonist Development Candidate