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Identification of chemokine receptor CCR4 antagonist

Ashok V. Purandare,* Aiming Gao, Honghe Wan, John Somerville, Christine Burke, Carrie Seachord, Wayne Vaccaro, John Wityak and Michael A. Poss

Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ 08543, USA

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Abstract—The present study reports the identification and hits to leads optimization of chemokine receptor CCR4 antagonists. Compound **12** is a high affinity, non-cytotoxic antagonist of CCR4 that blocks the functional activity mediated by the receptor.

Chemokines are a group of small (~8–14 kDa), mostly basic, structurally related cytokine peptides that regulate cell trafficking of various types of leukocytes through interactions with a subset of seven transmembrane G-protein coupled receptors.[1] CCR4 is a chemokine receptor that partners with the ligands MDC (macrophage derived chemokine, CCL22) and TARC (thymus and activation-regulated chemokine, CCL17), both of which are members of the beta or CC class of chemokines.[2] The CCR4 chemokine receptor is important in facilitating the migration of selected CD4 + thymocytes to the thymus and through the compartments of the thymus, as a part of the process of T cell maturation and differentiation. MDC, TARC, and CCR4 expressing Th2 cells are found in asthmatic lungs, arthritic joints, and inflamed skin. A CCR4 antagonist is expected to prevent recruitment of CD4 + Th2 polarized T cells to sites of inflammation by blocking chemotaxis and cellular activation. Anti-MDC and anti-TARC antibodies are each separately reported to have efficacy in murine asthma models.[3] Additional in vivo studies in animal models have demonstrated utility of these antibodies in preventing other immunological responses.[4] CCR4 antagonists are expected to have therapeutic potential in the treatment of diseases such as asthma, rheumatoid arthritis, and psoriasis.[1] Small molecule antagonists of CCR4 were recently disclosed.[5] Herein we report identification of a small molecule CCR4 antagonist, which blocks the functional response mediated through the receptor.

During the screening of the corporate compound collection, a series of closely related quinazoline, quinoline, and isoquinoline derivatives were identified as 'hits' with modest activity in the CCR4 binding assay (Fig. 1).[11] The compounds also inhibited chemotaxis. However, these hits were found to be cytotoxic to the cells. The observed inhibition of chemotaxis may be due to cytotoxicity and not because of receptor antagonism.

The foremost goal for the program was to determine if cytotoxicity and CCR4 antagonist activity could be diverged by structural modification of our initial hits. SAR from the screening suggested that the position of the ring nitrogen atoms in the central ring made little difference to the CCR4 activity. We embarked upon systematic exploration of linkers that separated the central aromatic core and the terminal aromatic moiety ($\mathbf{R^1}$). These analogs were synthesized from 2-aminobenzamide using a known approach as shown below (Scheme 1).[6] The overall yields of products varied from 35% to 60%.

As shown in Table 1, the two atoms tether either in the form of a straight chain (**4d**) or as a part of an aryl ether (**4f**) or as a part of a constrained ring (as in naphthalene,

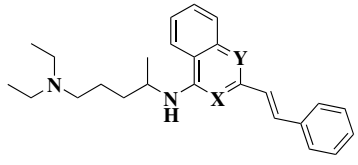

Figure 1. Either X or Y = N or both N; CCR4 (MDC binding) IC_{50} = 3.6–6.1 μM; cell cytotoxicity CC_{50} = 3–5 μM; chemotaxis Inh = 12–20 μM.

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* Corresponding author. Tel.: +1 609 252 4320; fax: +1 609 252 7446; e-mail: ashok.purandare@bms.com

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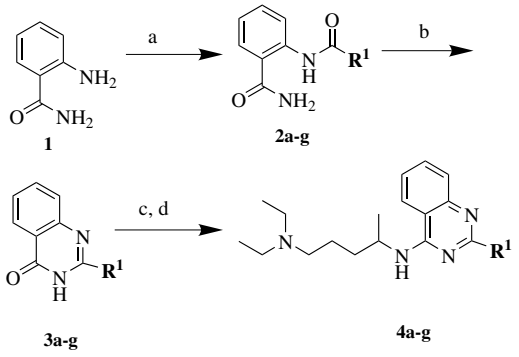
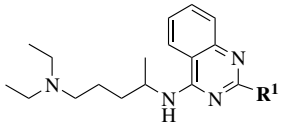


Scheme 1. Reagents and conditions: (a) R_1CO_2H, EDCI, CH_2Cl_2, 85%; (b) NaOEt, H_2O_2, 65%; (c) $POCl_3$, 100 °C; (d) N^1,N^1-diethyl-pentane-1,4-diamine, 95 °C, $(i\text{-Pr})_2$EtN, NMP, 50%.

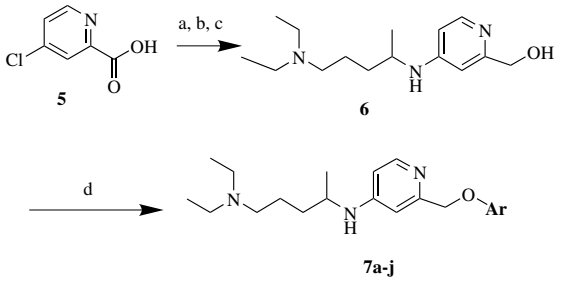
Table 1.



Compd #	R^1	CCR4 IC_{50} (μM)[11]
4a	Ph	> 25
4b	2-Naphthyl	**2.5**
4c	$-CH_2Ph$	10
4d	$-CH_2CH_2Ph$	**2.4**
4e	$-CH_2CH_2CH_2Ph$	>10
4f	$-CH_2OPh$	**2**
4g	–CONHPh	>10

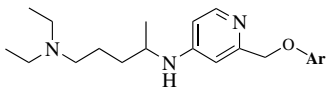
4b) was preferred.[7,11] On the contrary, an amide linker was not tolerated.

In addition to these changes, removal of the fused phenyl ring was also tolerated (data not shown). Since the position of the core nitrogen atoms did not affect the activity (observed from original hits), we decided to examine the nature of substitution pattern in the terminal aromatic group using a pyridine scaffold with benzyl ether system. The required pyridine scaffold was prepared in two steps from commercially available 4-chloro-pyridine-2-carboxylic acid (Scheme 2).[8] A parallel synthesis of aryl ether analogs was carried out using modified Mitsunobu conditions.[9] The summary of results is as shown in Table 2. 2,4-Dichlorophenyl was found to be the preferred terminal aromatic group (compound **7b**). These compounds were devoid of the cell toxicity (at 100 μM) observed in the original hits.



Scheme 2. Reagents and conditions: (a) EtOH, H_2SO_4, 90%; (b) N^1,N^1-diethyl-pentane-1,4-diamine, 95 °C, $(i\text{-Pr})_2$ EtN, NMP, 62%; (c) LAH, THF, 60 °C, 85%; (d) ArOH, ^{n}Bu$_3$P, 1,1′-(azodicarbonyl)-dipiperidine, CH_2Cl_2, 62%.

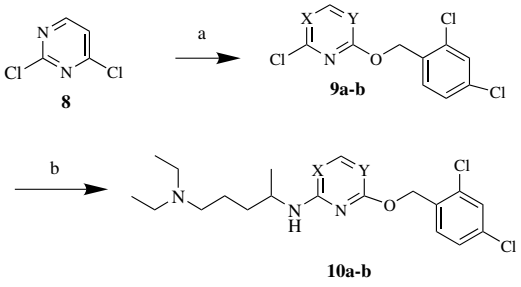
Table 2.



Compd #	Ar	CCR4 IC_{50} (μM)[11]
7a	2-Cl-Ph	17
7b	2,4-Di-Cl-Ph	**1.2**
7c	4-Cl-Ph	20
7d	3-Cl-Ph	25
7e	3,4-Di-Cl-Ph	4
7f	3,5-Di-Cl-Ph	25
7g	Ph	>30
7h	2,4-Di-OMe-Ph	>30
7i	3-OMe-Ph	>30
7j	4-F-Ph	18

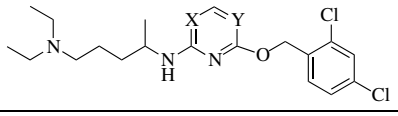
We then embarked on further examination of the linkage while keeping the Ar group constant (2,4-di-Cl-Ph). These compounds were synthesized by sequential displacement of chlorines from 4,6 dichloro-pyrimidine[10] (Scheme 3). As seen with compounds (**10a** vs **7b**), reversal of the ether linkage was tolerated. In addition, no dramatic difference in the activity of two regioisomers (**10a** and **b**) was observed (Table 3).

We subsequently explored the chain length that separates the terminal amino group and the core as well as the requirement of the amino group. As shown in the table, a linker with more than three atoms was required.



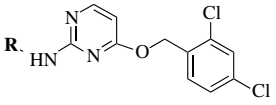
Scheme 3. Reagents and conditions: (a) 2,4-di-Cl-benzyl alcohol, ^{n}Bu$_4$NOH, chlorobenzene, separate regioisomers, 60% major; (b) N^1,N^1-diethyl-pentane-1,4-diamine, 95 °C, $(i\text{-Pr})_2$ EtN, NMP, 73%.

Table 3.



Compd #	X	Y	CCR4 IC_{50} (μM)[11]
10a	N	CH	1.5
10b	CH	N	2.8

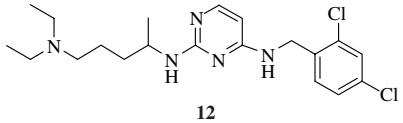
Table 4.



Compd #	R	CCR4 IC_{50} (μM)[11]
11a	–(CH)Me-$(CH_2)_3N(Et)_2$	1.5
11b	–$(CH_2)_3$-$N(Et)_2$	8
11c	–$(CH_2)_4$-$N(Et)_2$	3
11d	–$(CH_2)_2$-$N(Et)_2$	9
11e	–$(CH_2)_6$-$N(Me)_2$	2
11f	–(CH)Me-$(CH_2)_3CH(Me)_2$	>10

Also removal of the terminal nitrogen was found to be detrimental for the activity (compound **11f**) (Table 4).

Finally, replacement of oxygen as the linking atom with nitrogen was found to be optimum and this modification enhanced the activity by >5-fold. Compound **12** also showed significant inhibition of chemotaxis (IC_{50} = 5 μM) without cytotoxicity (CC50 > 100 M). Compound **12** also showed selectivity against CCR3 (IC_{50} = 2 μM vs eotaxin), CCR2 (IC_{50} > 10 μM vs MCP-1) and CXCR3 (IC_{50} > 30 μM vs I-TAC) in radioligand based binding assays.



12
CCR4IC_{50} 0.27 μM

In conclusion, we have identified a potent non-cytotoxic small molecule antagonist of CCR4 that showed inhibition of functional response mediated by binding of chemokine to the receptor. Further work related to the optimization of this chemotype will be reported in due course.

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7. All compounds were characterized by LC–MS and NMR analysis. In addition, the yields were based on weight of pure product unless mentioned otherwise.
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11. Assay conditions: CCR4 binding assay. A whole cell scintillation proximity assay (SPA) format was used for binding assays. HEK293 cells that were stably transfected with the human CCR4 receptor (accession # X85740), were suspended in assay buffer and plated at 4×10^4 cells/well into poly-L-lysine (100 μg/ml in PBS, 100 μl/well, O/N, 4 °C) treated solid white 96-well plates (Costar #3917). Assay buffer was phenol-red free DMEM (GIBCO #31053-028) supplemented with 10% FBS and 2 mM L-glutamine. Cells were incubated overnight at 37 °C, with 5% CO_2. Following incubation, 100 μl of binding mix was added to each well. Binding mix contains WGA-PVT SPA beads to give a 0.1 mg/well final concn, and ^{125}I-MDC [50 μC/ml] to give a 0.1 nM final concn, in binding buffer. Binding buffer is phenol-red free DMEM, 0.5% BSA (Sigma #A7284), and 2 mM L-glutamine. Compounds diluted in DMSO were added to the wells (1 μl/well, 1%) and 100 nM unlabeled MDC was added to non-specific control wells. The plate was sealed using Top Seal A (Packard) and the plate was mixed with slight agitation for 15 min at room temperature (rt). Following 24 h of incubation at rt without shaking, the plate was counted on a TopCount (Packard). Comparisons of SPA format results with direct receptor binding filter-based assays gave comparable results. Hill values for compound **12** ranged from 0.7 to 0.8 in five IC_{50} determinations. A typical binding assay would have a non-specific binding value from 100 cpm to 150 cpm, a signal to noise ratio between 8:1 and 16:1, and a signal window ranging from 5 to 20 (based on the number of standard deviations of the largest signal). Substitution of TARC in the binding assay for MDC gave IC_{50} values within 2-fold of the MDC based values. The HEK293/CCR4 cell line was determined to have 36,000 receptors per cell by Scatchard analysis.
Cytotoxicity assay: to test compounds for cytotoxicity, compounds were diluted as above to 2 × concentrations in phenol-red free RPMI supplemented with 10% FBS and Pen./Strep. at a volume of 50 μl/well. CCR4 transfected L1.2 cells (murine pre-B cell) grown in the same media, were harvested by centrifugation, and suspended in media at 2×10^6 cells/ml. Cells (50 μl) were then added to each well containing 2 × compound. The plate was mixed and incubated for 24 h at rt. For 0% viability controls, 6 μl/well of a 5% saponin solution was added to control wells

following incubation. The plate was then centrifuged for 5 min at 200 × g and the media aspirated. A 100 μl of a 16 μg/ml solution of calcein-AM (Molecular Probes #C-1430) in media was added and the plate incubated in the dark at rt for 30 min. The plate was then read in a Cytofluor 4000 (ex. = 485 nm, em. = 530 nm). L1.2 cells were found to be more sensitive to cytotoxic compounds than HEK293 cells in this assay.

Chemotaxis assay: CCR4 transfected L1.2 cells were grown in phenol-red free RPMI (GIBCO #11835-030) supplemented with 10% FBS and Pen./Strep. log phase cells were harvested by centrifugation, and washed in RPMI supplemented with 0.5% BSA (CA buffer). The cells were then suspended in CA buffer at 6×10^6 cells/ml. To these cells, calcein-AM (Molecular Probes #C-1430) was added to a final concentration of 10 μg/ml. The cells were incubated in the dark for 30 min at rt. Following incubation, 50 μl of cells were added to compounds that had been diluted to 2 × concentration in 50 μl CA buffer, and further incubated for 30 min. in the dark at rt. Chemotactic solutions of MDC at 100, 10, and 2 nM were prepared in CA buffer and were placed in the lower chambers of chemotaxis plates with 8 μm pores (Neuro-Probe #101-8). For 100% migration control wells, 25 μl of compound free cell suspension was placed in the lower position of the control wells and brought to volume with CA buffer. The chemotaxis filter was placed over the wells and 25 μl of the cell/compound suspensions were placed on the filter over each well. All samples were run in triplicate. The plate was then incubated for 1 h at 37 °C and 5% CO_2. Following incubation, the cells were gently removed from the top of the filter with a PBS wash and the plate was read on a Cytofluor 4000 (ex. = 485, em. = 530). Migration of at least 4% of the cells was required for consistent and reproducible results, with cell migrations typically in the 10–15% range. IC_{50} values were determined at the chemokine concentration that gave maximal cell migration (typically 2–5 nM with MDC).