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Discovery of INCB3344, a potent, selective and orally bioavailable antagonist of human and murine CCR2

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ABSTRACT

Rational design based on a pharmacophore of CCR2 antagonists reported in the literature identified lead compound **9a** with potent inhibitory activity against human CCR2 (hCCR2) but moderate activity against murine CCR2 (mCCR2). Modification on **9a** led to the discovery of a potent CCR2 antagonist **21** (INCB3344) with IC_{50} values of 5.1 nM (hCCR2) and 9.5 nM (mCCR2) in binding antagonism and 3.8 nM (hCCR2) and 7.8 nM (mCCR2) in antagonism of chemotaxis activity. INCB3344 exhibited > 100-fold selectivity over other homologous chemokine receptors, a free fraction of 24% in human serum and 15% in mouse serum, and an oral bioavailability of 47% in mice, suitable as a tool compound for target validation in rodent models.

The migration of leukocytes from blood vessels into inflamed tissues is involved in the initiation of a normal, disease-fighting, inflammatory response. The correct, controlled trafficking of these cells is an essential feature of the immune response to infection, but loss of control results in excessive recruitment of leukocytes to sites of inflammation, leading to onset and progression of a variety of chronic inflammatory diseases. Excessive recruitment of monocytes, a subset of leukocytes, and macrophages, which are derived from monocytes, to disease sites is especially harmful as these cells are well-characterized mediators of tissue destruction in chronic inflammatory and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis.[1,2] Accordingly, preventing or reducing migration of monocytes and macrophages to these disease sites would be expected to be beneficial in these diseases.

The infiltration of monocytes/macrophages into sites of inflammation is driven by monocyte chemoattractant protein-1 (MCP-1, CCL2) through interaction with its specific receptor, CCR2, which is a member of the super family of seven-transmembrane G-protein-coupled receptors (GPCRs) and is predominantly expressed on monocytes.[3] Binding of MCP-1 to CCR2 induces chemotaxis, resulting in directed migration of monocytes/macrophages to disease sites where MCP-1 expression is elevated.[4] Studies in rodent models by genetic deletion of either MCP-1[5] or CCR2[6–8] and use of peptidyl CCR2 antagonists[9] or anti-MCP-1 antibodies[10] have demonstrated the critical role of MCP-1/CCR2 in models of rheumatoid arthritis,[9,10] multiple sclerosis,[11–13] atherosclerosis,[8,14–16] and neuropathic pain,[17] and strongly suggest that CCR2 is an attractive target for potential therapeutic intervention in these

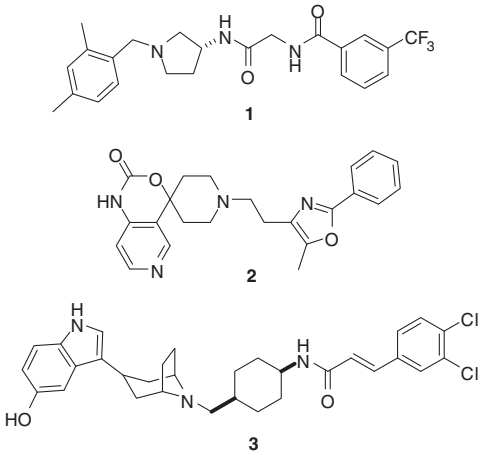

Figure 1. CCR2 antagonists reported in the literature.

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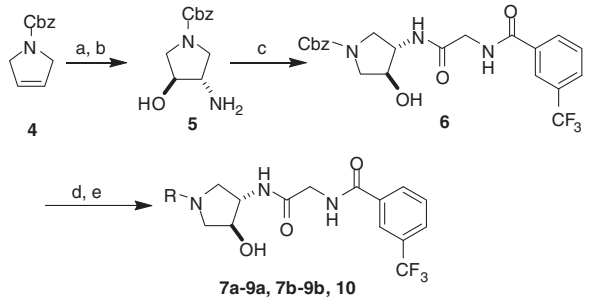
diseases. As a result, inhibition of CCR2 has emerged as a novel therapeutic approach for pharmaceutical research.

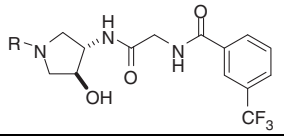
Despite the many publications on small molecule CCR2 antagonists,[18–29] only three classes of CCR2-selective antagonists had been disclosed at the time we initiated a CCR2 antagonist discovery program (Fig. 1). They include Teijin compound **1**,[30] Roche compound **2**[31] and SmithKline Beecham compound **3**,[32,33] all exhibiting moderate inhibitory activity toward human CCR2 (hCCR2). A tertiary amine in the central region and two lipophilic moieties at the ends of each molecule represent the common pharmacophore among these inhibitors. As demonstrated by compound **2**, the basic amine is an important contributor to the binding affinity of these molecules through a putative salt bridge interaction with an acidic residue in transmembrane region 7 of the receptor, which is found in most chemokine receptors.[31] Since the aromatic ring on the left-hand side and the tertiary amine are separated by one carbon in compound **1** and by three carbons in compounds **2** and **3**, we reasoned that separation of the aromatic ring and the tertiary amine in **1** with a carbocycle or a saturated heterocycle should increase the basicity of the nitrogen. This accordingly should strengthen the salt bridge interaction with the receptor, potentially leading to an enhancement of binding affinity if an optimal orientation of the aromatic ring on the carbocycle or saturated heterocycle could be defined. Based on this consideration, we designed scaffolds A, B and C in which an OH at the 4-position of the pyrrolidine is intended for further SAR exploration (Fig. 2).

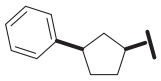
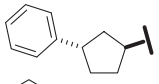
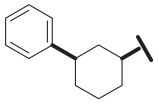
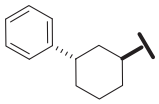
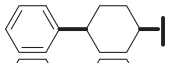
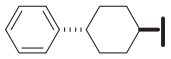
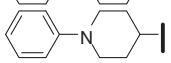
To quickly test our design, we synthesized compounds **7a–b**, **8a–b**, **9a–b** and **10** (Scheme 1). The synthesis started with the commercially available benzyl dihydropyrrol-1-carboxylate **4**. Epoxidation of **4** using mCPBA followed by ring opening with ammonium hydroxide provided trans-1-Cbz-3-hydroxy-4-aminopyrrolidine **5** as a racemic mixture. BOP coupling of **5** with *N*-(3-trifluoromethylbenzoyl)glycine, which was prepared by reaction of glycine with 3-trifluoromethylbenzoyl chloride in aqueous sodium hydroxide and THF in one step, gave rise to compound **6**. Following removal of the Cbz group in **6** by hydrogenation, reductive amination of the resulting pyrrolidine with a substituted cyclic ketone using sodium triacetoxyborohydride afforded the products **7a–b**, **8a–b** and **9a–b** as a mixture of cis and trans isomers on the carbocycles, with a ratio ranging from 3:1 to 5:1, and the product **10**. Separation of the cis and trans mixture in **7a–b**, **8a–b** and **9a–b** by flash chromatography or by reversed phase HPLC afforded the major isomers **7a–9a** and the minor isomers **7b–9b**. The major isomers were assigned as the cis isomers based on the employed chemistry in which the reducing agent used for reductive amination was the bulky sodium triacetoxyborohydride. This assignment was confirmed by 2-dimensional NMR studies.



Scheme 1. Reagents and conditions: (a) mCPBA, CH_2Cl_2; (b) ammonium hydroxide, 70% for two steps; (c) *N*-(3-trifluoromethyl)benzoylglycine, BOP, Et_3N, DMF, 80%; (d) Pd/C, MeOH, 100%; (e) ketone, $Na(OAc)_3BH$, THF.

Table 1
Compounds **7a–b**, **8a–b**, **9a–b** and **10**



Compd	R	hCCR2[a] IC_{50} (nM)	
		MCP-1	CTX
7a		120	
7b		>1000	
8a		>1000	
8b		>1000	
9a		7.4	12
9b		>1000	
10		649	

[a] Human MCP-1 binding assay and chemotaxis assay.

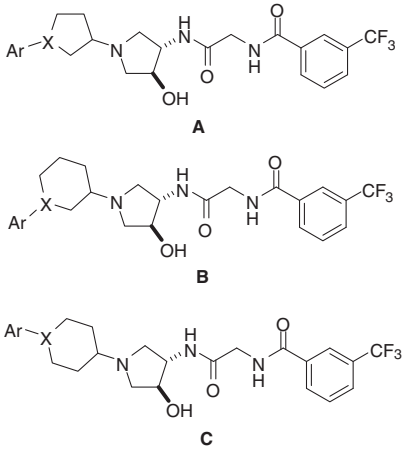


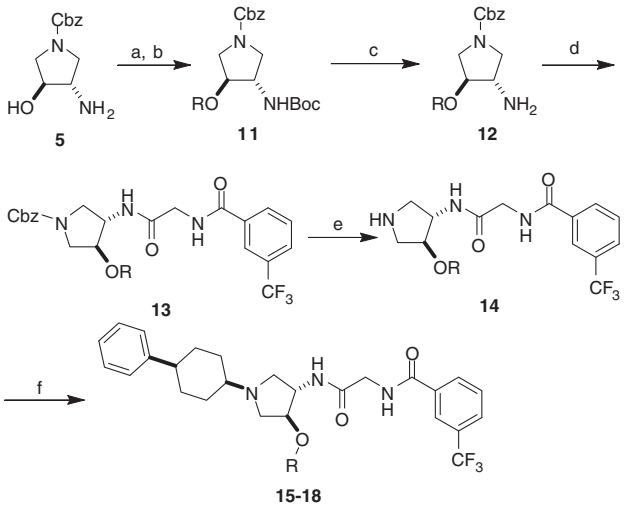
Figure 2. Scaffolds A, B and C.

Compounds were screened by binding assay to assess their ability to inhibit MCP-1 binding to human peripheral blood monocytes (MCP-1 assay)[34] and by a functional assay to determine their ability to inhibit MCP-1 stimulated chemotaxis in human peripheral blood monocytes (CTX assay).[35] As shown in Table 1, in the cyclopentane series, the cis isomer **7a** is a moderate hCCR2 inhibitor with an IC_{50} of 120 nM in the MCP-1 binding assay while the trans isomer **7b** is inactive in the same assay (IC_{50} >1 μM). In the cyclohexane series, both the cis and trans isomers **8a–b** showed no inhibitory activity with IC_{50} values of >1 μM when the phenyl residue is at the 3-position on the cyclohexyl. In contrast, when the phenyl residue is at the 4-position on the cyclohexyl, the cis isomer **9a** is a potent hCCR2 inhibitor with IC_{50} values of 7.4 nM in the MCP-1 assay and 12 nM in the chemotaxis assay while the trans isomer **9b** is inactive (IC_{50} >1 μM). By comparison, the piperidine analog **10** in which the

phenyl residue at the 1-position of piperidine predominantly assumes the more stable trans orientation relative to the pyrrolidine substituent at the 4-position is a weak inhibitor (IC_{50} = 649 nM). These results revealed that the geometric alignment and orientation of the phenyl ring relative to the pyrrolidine ring exert a significant impact on the interaction of the molecule with the CCR2 receptor. The optimal geometric alignment of the phenyl ring and the pyrrolidine ring is at 1- and 4-positions on a six-membered carbocycle and the optimal orientation of the phenyl ring is cis relative to the pyrrolidine ring.

One of our main goals at the early stage of the CCR2 program was to identify a tool compound with high affinity for murine CCR2 (mCCR2), high selectivity over other homologous chemokine receptors and good oral bioavailability suitable for target validation in rodent models. The potent anti-hCCR2 activity of **9a** prompted us to run a screen using a murine monocytic cell line that expresses CCR2.[36] Encouragingly, **9a** displayed a moderate anti-mCCR2 activity, with IC_{50} values of 186 and 265 nM in MCP-1 and chemotaxis assays, respectively.

To improve the anti-mCCR2 activity of **9a**, we first conducted SAR investigation at the hydroxyl by introducing an alkyl group. Attempts to directly alkylate the hydroxyl in **9a** with alkyl bromide or iodide using NaH in DMF resulted in multiple products. As a result, alkylation was carried out following the protection of the amine in **5** with a Boc (Scheme 2). After removal of the Boc in the alkylated product **11** using HCl in dioxane, the resulting amine **12** was coupled with the glycine residue to give intermediate **13**. Removal of Cbz by hydrogenation was followed by reductive amination of the resulting amine **14** with 4-phenylcyclohexanone to provide a mixture of cis and trans isomers. The major cis isomers were separated by flash chromatography to give target compounds **15**–**18**.

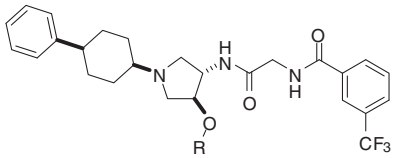


Scheme 2. Reagents and conditions: (a) $(Boc)_2O$, Et_3N, THF, 90%; (b) alkyl halide, NaH, DMF, 0 °C; (c) 4 N HCl/dioxane; (d) *N*-(3-trifluoromethyl)benzoylglycine, BOP, Et_3N, DMF; (e) Pd/C, MeOH; (f) 4-phenylcyclohexanone, $Na(OAc)_3BH$, THF.

Alkylation at the hydroxyl in **9a** with ethyl (**15**) improved the hCCR2 binding affinity from an IC_{50} of 7.4 nM to an IC_{50} of 0.95 nM and the chemotaxis activity from an IC_{50} of 12 nM to an IC_{50} of 6.5 nM (Table 2). Importantly, the mCCR2 activity was more dramatically enhanced by this modification, with 98-fold improvement in binding affinity and 19-fold improvement in chemotaxis activity. Compound **15** is now a potent mCCR2 antagonist with IC_{50} values of 1.9 nM in binding affinity and 14 nM in chemotaxis activity. Replacement of the ethyl group in **15** with longer alkyl groups resulted in a loss in either chemotaxis activity (*n*-propyl in **16**) or binding affinity (*n*-butyl in **17** and benzyl in **18**). Despite its potent antagonistic activity against mCCR2, however, compound **15** is highly protein bound, with a free fraction of 0.5% in human serum and 2.3% in mouse serum. In addition, compound **15** exhibited a potent hERG binding activity, with an IC_{50} of <1 μM in a dofetilide binding assay.

To refine the protein binding and hERG activity in **15**, we turned our SAR studies to the hydrophobic moiety of 4-phenylcycohexyl by introducing a polar group such as hydroxyl to the sterically hindered 4-position of the cyclohexyl. Introduction of a hydroxyl group at the benzylic position on the cyclohexyl in **15** to give **19** led to a significant enhancement in free fraction in protein binding and a dramatic drop in hERG binding activity (Table 3). The free fraction was improved to 25% in **19** from 0.5% in **15** in human serum and to 18% in **19** from 2.3% in **15** in mouse serum. The hERG binding activity was reduced to an IC_{50} of 17 μM in **19** from an IC_{50} of <1 μM in **15** in the dofetilide assay. Unfortunately, despite its suitable profile in protein binding and hERG binding activity, compound **19** is not potent enough as a tool compound for model studies in rodents. While the potent hCCR2 inhibitory activity was retained from this modification, a fivefold loss in mCCR2 binding affinity and 32-fold loss in mCCR2 chemotaxis activity accompanied the introduction of the polar hydroxyl group, indicating that there is no preference in polarity for hCCR2 while lipophilicity is preferred for mCCR2 at this position.

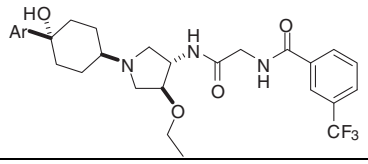
Table 2
Compounds **9a** and **15**–**18**



Compd	R	hCCR2[a] IC_{50} (nM)		mCCR2[b] IC_{50} (nM)		h/m PB[c]% free	hERG[e] IC_{50} (μM)
		MCP-1	CTX	MCP-1	CTX		
9a	H	7.4	12	186	265	2.6/ND[d]	<1
15	Ethyl	0.95	6.5	1.9	14	<0.5/2.3	<1
16	*n*-Propyl	2.9	1.8	1.9	158		
17	*n*-Butyl	47		54			
18	Benzyl	17		51			

[a] Human MCP-1 binding assay and chemotaxis assay.
[b] Murine MCP-1 binding assay and chemotaxis assay.
[c] % free fraction in human and mouse protein binding.
[d] Not determined.
[e] hERG dofetilide binding activity.

Table 3
Compounds **15** and **19–21**

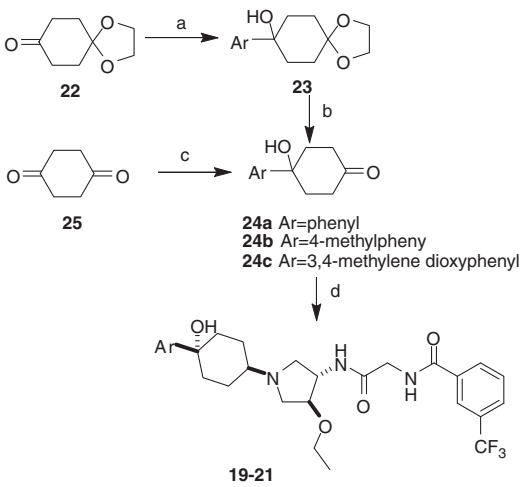


Compd	Ar	hCCR2[a] IC_{50} (nM)		mCCR2[b] IC_{50} (nM)		h/mPB[c]% free	hERG[e] IC_{50} (μM)
		MCP-1	CTX	MCP-1	CTX		
15		0.95	6.5	1.9	14	0.5/2.3	<1
19	Phenyl	2.3	3	10	444	26/18	17
20	4-Methylphenyl	2.3	1.4	2.1	12.9	ND[d]/7.9	11
21	3,4-Methylenedioxyphenyl	5.1	3.8	9.5	7.8	24/15	13

[a] Human MCP-1 binding assay and chemotaxis assay.
[b] Murine MCP-1 binding assay and chemotaxis assay.
[c] % free fraction in human and mouse protein binding.
[d] Not determined.
[e] hERG dofetilide binding activity.

To improve the anti-mCCR2 activity in **19**, we fine-tuned the polarity of the 4-hydroxyl-4-phenylcyclohexyl moiety by modification at the phenyl ring. Substitution with methyl (**20**) at the 4-position on the phenyl in **19** improved the mCCR2 binding affinity and chemotaxis activity to IC_{50} values of 2.1 and 12.9 nM from IC_{50} values of 10 and 444 nM, respectively, but reduced the free fraction in mouse serum by twofold (Table 3). In contrast, substitution with methylenedioxy at the 3,4-position on the phenyl in **19** afforded a potent mCCR2 inhibitor **21** (INCB3344) with retained free fraction in mouse serum. INCB3344 exhibited IC_{50} values of 5.1 nM (hCCR2) and 9.5 nM (mCCR2) in binding affinity and 3.8 nM (hCCR2) and 7.8 nM (mCCR2) in chemotaxis activity. In protein binding, it had a free fraction of 24% in human serum and 15% in mouse serum. It showed a moderate hERG binding activity, with an IC_{50} of 13 μM in the dofetilide binding assay.

Compounds **19–21** were synthesized according to Scheme 3.[37] Addition of commercially available phenylmagnesium bromide or *p*-tolylmagnesium bromide to hexan-1,4-dione monoethylene ketal **22** gave rise to intermediate **23**. Treatment of **23** with aqueous HCl generated 4-phenyl-4-hydroxycyclohexanone **24a** and 4-(*p*-tolyl)-4-hydroxycyclohexanone **24b**. Under this condition, the hydroxyl group was unstable when the aryl residue was 3,4-methylenedioxyphenyl, leading to formation of a significant amount of dehydration product. As a result, compound **24c** was prepared in low yield by direct addition of 3,4-methylenedioxyphenylmagnesium bromide to cyclohexan-1,4-dione **25**. Reductive amination of **24a–c** with intermediate **14** generated a mixture of cis and trans isomers, with the smaller hydroxyl group (relative to aryl) trans to the pyrrolidine moiety being the major isomer (trans:cis = ~2:1). Separation of the two isomers by flash chromatography provided the major trans isomers **19–21**. The corresponding minor cis isomers showed IC_{50} values of >1 μM in the MCP-1 assay.



Scheme 3. Reagents and conditions: (a) Phenylmagnesium bromide or *p*-tolylmagnesium bromide, THF, −78 °C; (b) 2 N HCl in water; (c) 3,4-methylenedioxyphenylmagnesium bromide, THF, −78 °C, 25%; (d) **14**, $Na(OAc)_3BH$, THF.

Further in vitro profiling revealed that INCB3344 is a potent antagonist towards rat and cynomolgus CCR2 as well, displaying IC_{50} values of 7.3 and 16 nM in binding antagonism and 2.7 and 6.2 nM in antagonism of chemotaxis activity, respectively. INCB3344 is a selective hCCR2 antagonist, exhibiting IC_{50} values of more than 1 μM against a panel of >50 ion channels, transporters, chemokine receptors and other selected GPCRs. It is also a selective mCCR2 antagonist, showing IC_{50} values of >1 μM and >3 μM against murine CCR1 and murine CCR5, respectively, the two most homologous chemokine receptors to mCCR2. Other in vitro and in vivo pharmacological properties of INCB3344 have been characterized and reported.[36,38]

The pharmacokinetics of INCB3344 was assessed in both CD-1 and Balb/c mice (Table 4). When administered intravenously to CD-1 mice, INCB3344 exhibited a high clearance and a moderate volume of distribution, resulting in a short half life of 1 h. Despite its high clearance, however, good oral exposure was achieved, with an AUC at 2664 nM h at a dose of 10 mg/kg. The oral bioavailability was 47%. By comparison, slightly better oral exposure (AUC = 3888 nM h) was obtained when administered orally to Balb/c mice at the same dose. This PK property, coupled with its potent anti-mCCR2 activity and good selectivity, made this compound suitable for model studies in rodents.

INCB3344 has been used as a tool compound for target validation in a mouse model of multiple sclerosis, a rat model of inflammatory arthritis and a mouse model of obesity, and was effective in

Table 4
Pharmacokinetic parameters of INCB3344

Mouse strain		CD-1	Balb/c
Dose iv/po (mg/kg)		5/10	/10
iv	CL (L/h/kg)	5.1	
	V_{dss} (L/h)	2.8	
	$T_{1/2}$ (h)	1.0	
po	C_{max} (nM)	1886	961
	AUC (nM h)	2664	3888
	F (%)	47	

lowering macrophage content in target tissues and in suppressing development of these disease models.[36,39] The results from these studies delineated for the first time the pharmacological relevance of inhibiting CCR2 using a small molecule antagonist in disease models.

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34. Human PBMCs were used to test compounds in a binding assay. 200,000 to 500,000 cells were incubated with 0.1–0.2 nM ^{125}I-labeled MCP-1, with or without unlabeled competitor (10 nM MCP-1) or various concentrations of compounds to be tested. ^{125}I-labeled MCP-1, were prepared by suitable methods or purchased from commercial vendors (Perkin Elmer, Boston, MA). The binding reactions were performed in 50–250 μL of a binding buffer consisting of 1 M HEPES pH 7.2, and 0.1% BSA (bovine serum albumin), for 30 min at room temperature. The binding reactions were terminated by harvesting the membranes by rapid filtration through glass fiber filters (Perkin Elmer) which was presoaked in 0.3% polyethyleneimine or Phosphate Buffered Saline (PBS). The filters were rinsed with approximately 600 μL of binding buffer containing 0.5 M NaCl or PBS, then dried, and the amount of bound radioactivity was determined by counting on a Gamma Counter (Perkin Elmer).
35. The capacity of compounds to antagonize CCR2 function was determined in a leukocyte chemotaxis assay using human peripheral blood mononuclear cells, in a modified Boyden Chamber (Neuro Probe). 500,000 cells in serum free DMEM media (In Vitrogen) were incubated with or without the inhibitors and warmed to 37 °C. The chemotaxis chamber (Neuro Probe) was also prewarmed. 400 μL of warmed 10 nM MCP-1 was added to the bottom chamber in all wells except the negative control which had DMEM added. An 8 micron membrane filter (Neuro Probe) was placed on top and the chamber lid was closed. Cells were then added to the holes in the chamber lid which were associated with the chamber wells below the filter membrane. The whole chamber was incubated at 37 °C, 5% CO_2 for 30 min. The cells were then aspirated off, the chamber lid opened, and the filter gently removed. The top of the filter was washed three times with PBS and the bottom was left untouched. The filter was air dried and stained with Wright Geimsa stain (Sigma). Filters were counted by microscopy. The negative control wells served as background and were subtracted from all values. Antagonist potency was determined by comparing the number of cells that migrated to the bottom chamber in wells which contained antagonist, to the number of cells which migrated to the bottom chamber in MCP-1 control wells.
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37. Experimental procedures for compound **21** (INCB3344):
Benzyl 6-Oxa-3-azabicyclo[3.1.0]hexane-3-carboxylate. To a solution of benzyl 3-pyrroline-1-carboxylate (30 g, 133 mmol) in CH_2Cl_2 (700 mL) was added mCPBA (57.2 g, 200 mmol). The reaction mixture was stirred at room temperature overnight and quenched with 20% $NaHSO_3$ aqueous solution (250 mL). The organic phase was separated, and the aqueous layer was extracted with CH_2Cl_2 twice. The combined extracts were washed with $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated under reduced pressure. Chromatography on silica gel eluting with 40% EtOAc/hexanes provided the title compound (24 g, 83%). MS $(M+H)^+$ 220.
Benzyl trans-3-[(tert-Butoxycarbonyl)amino]-4-hydroxypyrrolidine-1-carboxylate. To a solution of benzyl 6-oxa-3-azabicyclo[3.1.0]hexane-3-carboxylate (20.7 g, 94.4 mmol) in 80 mL of methanol was added ammonium hydroxide (80 mL). The reaction mixture was stirred at 60 °C overnight and concentrated under reduced pressure to give an oil residue (22.3 g, 94.4 mmol) which was used directly for the next reaction. MS: $(M+H)^+ = 237$.
To a solution of the above crude product in THF (200 mL) was added di-*tert*-butyldicarbonate (26.8 g, 123 mmol) and triethylamine (17.1 mL, 123 mmol) at 0 °C. The reaction mixture was stirred at room temperature overnight. After addition of EtOAc and water, the organic phase was separated, and the aqueous layer was extracted with EtOAc. The combined extracts were washed with $NaHCO_3$ and brine, dried over Na_2SO_4, and evaporated under reduced pressure. Chromatography on silica gel eluting with 70% EtOAc/hexanes provided the title compound (27.3 g, 86%). MS: $(M+H)^+ = 337$. ^{1}H NMR (400 MHz, CD_3OD): δ ppm 7.34 (m, 5H), 5.10 (s, 2H), 4.10 (m, 1H), 3.86 (m, 1H), 3.70 (m, 1H), 3.58 (m, 1H), 3.36 (m, 2H), 1.40 (s, 9H).
Benzyl trans-3-ethoxy-4-[(tert-butoxycarbonyl)amino]pyrrolidine-1-carboxylate. To a solution of benzyl 3-[(*tert*-butoxycarbonyl)amino]-4-hydroxypyrrolidine-1-carboxylate (26 g, 77 mmol) in THF (120 mL) was added NaH (5 g, 211 mmol) at 0 °C. The reaction mixture was stirred at 0 °C for 1 h and iodoethane (17.9 g, 115 mmol) was added. After being stirred at room temperature overnight, water and EtOAc were added. The organic phase was separated, and the aqueous layer was extracted with EtOAc twice. The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. Chromatography on silica gel eluting with 25% EtOAc/hexanes provided the title compound (19.6 g, 70%). MS: $(M+H)^+ = 365.1$. ^{1}H NMR (400 MHz, CD_3OD): δ ppm 7.35 (m, 5H), 5.12 (s, 2H), 4.00 (m, 1H), 3.82 (m, 1H), 3.60 (m, 4H), 3.40 (m, 2H), 1.42 (s, 9H), 1.18 (t, J = 6.8 Hz, 3H).

Benzyl trans-3-ethoxy-4-[({[3-(trifluoromethyl)benzoyl]amino}acetyl)amino] pyrrolidine-1-carboxylate. To a solution of benzyl 3-ethoxy-4-[(*tert*-butoxycarbonyl)amino]pyrrolidine-1-carboxylate (18.2 g, 50 mmol) in THF (100 mL) was added a solution of 4 N HCl in dioxane (250 mL). After being stirred at room temperature for 2 h, the solution was concentrated under reduced pressure. The residue was dissolved in saturated $NaHCO_3$ solution. The resulting solution was extracted with EtOAc twice. The combined extracts were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure to give benzyl *trans*-3-ethoxy-4-aminopyrrolidine-1-carboxylate as an oil. MS: $(M+H)^+ = 265.1$.

To a solution of benzyl *trans*-3-ethoxy-4-aminopyrrolidine-1-carboxylate (9.5 g, 36 mmol) in DMF (100 mL) in an ice bath was added *N*-methylmor pholine (12 g, 105 mmol), BOP (19 g, 44 mmol) and *N*-[(3-trifluoromethyl) benzoyl]glycine (10 g, 39 mmol). After being stirred at room temperature overnight, the solution was diluted with EtOAc. The resulting solution was washed with $NaHCO_3$ and brine, dried ($MgSO_4$) and concentrated. Chromatography on silica gel eluting with 50% EtOAc-hexanes provided the title compound (14.2 g, 80%). MS: $(M+H)^+ = 494.1$. ^1H NMR (400 MHz, CD_3OD): δ ppm 8.20 (s, 1H), 8.11 (d, $J = 7.6$ Hz, 1H), 7.85 (d, $J = 8$ Hz, 1H), 7.68 (t, $J = 8$ Hz, 1H), 7.35 (m, 5H), 5.12 (s, 2H), 4.35 (m, 1H), 4.02 (s, 2H), 3.95 (m, 1H), 3.60 (m, 4H), 3.42 (m, 2H), 1.17 (t, $J = 6.8$ Hz, 3H).

4-(Benzo[d][1,3]dioxol-5-yl)-4-hydroxycyclohexanone. To a solution of 1,4-cyclohexanedione (11.2 g, 100 mmol) in THF (200 mL) cooled at −78 °C was added a 1 M solution of 3,4-(methylenedioxy)phenylmagnesium bromide in toluene/THF (50:50) (50 mL, 50 mmol). The solution was allowed to warm to room temperature. After being stirred at room temperature for 3 h, the reaction was quenched with a solution of NH_4Cl. The resulting solution was extracted with EtOAc three times. The combined extracts were washed with brine, dried over $MgSO_4$ and concentrated. Purification on silica gel eluting with 50% EtOAc/hexanes yielded 2.35 g (20%) of the title compound. MS: $(M+H-H_2O)^+ = 217.1$.

trans-N-(2-{1-[4-(Benzo[d][1,3]dioxol-5-yl)-4-hydroxycyclohexyl]-4-ethoxypyrrolidin-3-ylamino}-2-oxoethyl)-3-(trifluoromethyl)benzamide (**21**, INCB3344). To a solution of benzyl *trans*-3-(ethoxy)-4-[({[3-(trifluoromethyl) benzoyl]amino} acetyl)amino]pyrrolidine-1-carboxylate (4.9 g, 10 mmol) in 50 mL of MeOH was added 10% Pd on carbon (250 mg) under nitrogen. The reaction mixture was stirred at room temperature under hydrogen overnight and filtered through Celite. The filtrate was concentrated under reduced pressure to give *trans*-*N*-[2-(4-ethoxypyrrolidin-3-ylamino)-2-oxoethyl]-3-(trifluoromethyl)benzamide (3.5 g, 97%). MS $(M+H)^+$ 360.1.

To a solution of 4-(benzo[*d*][1,3]dioxol-5-yl)-4-hydroxycyclohexanone (281 mg, 1.2 mmol) and *trans*-*N*-[2-(4-ethoxypyrrolidin-3-ylamino)-2-oxoethyl]-3-(trifluoromethyl)benzamide (360 mg, 1 mmol) in THF (10 mL) was added $Na(OAc)_3BH$ (424 mg, 2 mmol). The mixture was stirred at room temperature overnight and poured into a brine solution. The resulting solution was extracted with EtOAc twice. The combined extracts were washed with $NaHCO_3$ and brine, dried over $MgSO_4$ and concentrated. Purification on silica gel eluting with 5% MeOH/CH_2Cl_2 gave 230 mg of the fast moving isomer **21** with 99% purity (*trans* isomer, MS: $(M+H)^+ = 578.2$) and 120 mg of the slow moving isomer (*cis* isomer, MS: $(M+H)^+ = 578.2$). ^1H NMR of **21** (400 MHz, CD_3OD): δ ppm 8.20 (s, 1H), 8.11 (d, $J = 7.6$ Hz, 1H), 7.85 (d, $J = 8$ Hz, 1H), 7.67 (t, $J = 8$ Hz, 1H), 7.02 (s, 1H), 6.99 (d, $J = 8.0$ Hz, 1H), 6.73 (d, $J = 8$ Hz, 1H), 5.90 (s, 2H), 4.25 (m, 1H), 4.02 (s, 2H), 3.85 (m, 1H), 3.62 (m, 1H), 3.45 (m, 1H), 3.02 (m, 1H), 2.96 (m, 1H), 2.50 (m, 2H), 2.22 (m, 3H), 1.94 (m, 2H), 1.52 (m, 4H), 1.18 (t, $J = 6.8$ Hz, 3H).

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