Nicotinamide Glycolates Antagonize CXCR2 Activity through an Intracellular Mechanism

Dean Y. Maeda, Mark T. Quinn, Igor A. Schepetkin, Liliya N. Kirpotina, and John A. Zebala

Syntrix Biosystems, Inc., Auburn, Washington (D.Y.M., J.A.Z.); Department of Veterinary Molecular Biology, Montana State University, Bozeman, Montana (M.T.Q., I.A.S., L.N.K.); and Department of Laboratory Medicine, University of Washington, Seattle, Washington (J.A.Z.)

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ABSTRACT

The chemokine receptors CXCR1/2 are involved in a variety of inflammatory diseases, including chronic obstructive pulmonary disease. Several classes of allosteric small-molecule CXCR1/2 antagonists have been developed. The data presented here describe the cellular pharmacology of the acid and ester forms of the nicotinamide glycolate pharmacophore, a potent antagonist of CXCR2 signaling by the chemokines CXCL1 and CXCL8. Ester forms of the nicotinamide glycolate were inactive in whole-cell assays but they were inactive in cell-free assays of \(^{125}\text{I}\)-CXCL8/CXCR2 binding and CXCL1-stimulated guanosine 5'-O-[\(^{35}\text{S}\)]triphosphate (\(^{35}\text{S}\)GTP\(\gamma\)S) exchange. Acid forms of the nicotinamide glycolate were inactive in whole-cell assays of chemotaxis and calcium flux, but they inhibited \(^{125}\text{I}\)-CXCL8/CXCR2 binding and CXCL1-stimulated \(^{35}\text{S}\)GTP\(\gamma\)S exchange. The \(^3\text{H}\) ester was internalized by neutrophils and rapidly converted to the \(^3\text{H}\) acid in a concentrative process. The \(^3\text{H}\) acid was not internalized by neutrophils but was sufficient alone to inhibit CXCL1-stimulated calcium flux in neutrophils that were permeabilized by electroporation to permit its direct access to the cell interior. Neutrophil efflux of the acid was probenecid-sensitive, consistent with an organic acid transporter. These data support a mechanism wherein the nicotinamide glycolate ester serves as a lipophilic precursor that efficiently translocates into the intracellular neutrophil space to liberate the active acid form of the pharmacophore, which then acts at an intracellular site. Rapid inactivation by plasma esterases precluded use in vivo, but the mechanism elucidated provided insight for new nicotinamide pharmacophore classes with therapeutic potential.

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ABBREVIATIONS: PMN, polymorphonuclear leukocyte; CXC, Cys-Xaa-Cys; SB265610, [1-(2-bromo-phenyl)-3-(7-cyano-3H-benzotriazol-4-yl)-urea]; SCH527123, [2-hydroxy-N,N-dimethyl-3-[2-[1-[1(R)-5-methyl-2-furany]propyl]amino]-3,4-dioxo-1-cyclobuten-1-yl][amino]benzamide]; CCR1/2/3/9, chemokine (C-C motif) receptor 9; CXCL1/2/3, growth-related oncogenes \(\alpha\), \(\beta\), and \(\gamma\) (GRO \(\alpha\), \(\beta\)/\(\gamma\)); CXCL5, epithelial-derived neutrophil-activating peptide 78 (ENA-78); CXCL6, granulocyte chemotactic protein-2; CXCL7, neutrophil-activating protein-2 (NAP-2); CXCL8, interleukin-8; OAT, organic anion transporter; HBSS, Hanks’ balanced salt solution; HBSS\(\text{'}\), HBSS without Ca\(^{2+}\) and Mg\(^{2+}\); HPLC, high-performance liquid chromatography; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; GTP\(\gamma\)S, guanosine 5'-O-(3-thio)triphosphate; ANOVA, analysis of variance; AMD3100, 1,1',1-[4-phenylenediis(methylene)bis[1,4,8,11-tetraazacyclotetradecane] octohydrobromide dehydrate]; BX471, N-(5-chloro-2-(2-(4-(4-fluorophenyl)mercury)-1-methyl-1-piperazinyl)-2-oxoethoxy) phenylurea hydrochloric acid; TAK-799, (N,N-dimethyl-N-[4-[[[2-(4-methylphenyl)-6,7-dihydro-2H-benzocyclohepten-8-yl]carbonyl][amino][benzyl]-tetrahydro-2H-pyranyl-4-aminium chloride]; Compound 1, [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl] acetic acid methyl ester; Compound 2, [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl] acetic acid; Compound 3, [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl] acetic acid methyl ester; Compound 4, [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-yloxy]-acetic acid methyl ester; Compound 5, [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl] acetic acid benzyl ester; and Compound 6, [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ysulfanyl] acetic acid tert-butyl ester; Compound 7, 5,6-dichloro-N-(4-fluorophenyl)-nicotinamide; Compound 8, [3-chloro-5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ysulfanyl] acetic acid methyl ester; Compound 9, [3-chloro-5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ysulfanyl] acetic acid.
served cysteines. Specifically, the Glu-Leu-Arg motif-containing CXC chemokines are major PMN chemoattrac-
tants and activate these cells via two G protein-coupled CXC receptors (CXCRs) known as CXCR1 and CXCR2 (Holmes et al., 1991; Murphy and Tiffany, 1991; Cerretti et al., 1993; Geiser et al., 1993). CXCR1 preferentially binds interleukin-8 (CXCL8) but can also bind granulocyte che-
mothactic protein-2 (CXCL6) with lower affinity. By com-
parison, CXCR2 binds growth-related oncogenes α, β, and γ (CXCL1/2/3), epithelial-derived neutrophil-activating peptide 78 (CXCL5), neutrophil-activating protein-2 (CXCL7), CXCL6, and CXCL8.

CXCR1, CXCR2, and their chemokine ligands are involved in a wide range of acute and chronic inflammatory diseases, including acute respiratory distress syndrome (Kurdowska et al., 2002), rheumatoid arthritis (Erdem et al., 2005; Lally et al., 2005), psoriasis (Reich et al., 2001), inflammatory bowel disease (Banks et al., 2003), chronic obstructive pulmonary disease and asthma (Keatings et al., 1996; Beeh et al., 2003; Chapman et al., 2009), cystic fibrosis (Koller et al., 1997), and atherosclerosis (Bizzarri et al., 2006). CXCR1 and CXCR2 have thus garnered much attention as targets for small-
molecule drug discovery and have prompted the development of several potent small-molecule antagonists, several of which have progressed into clinical trials. These include the CXCR1-selective antagonist repertaxin ([R]−)-2-(4-isobutyl-
phenyl)propionyl methanesulfonamide (Bertini et al., 2004; Allegretti et al., 2005) and a series of CXCR2-selective di-
arylamides, as exemplified by SB265610 (White et al., 1998; Auten et al., 2001; Podolin et al., 2002). Derivatives of the latter series, where the urea was replaced with the 3,4-
diaminocyclobut-3-ene-1,2-dione bioisostere, led to antago-
nists with picomolar affinity for CXCR2, such as SCH527123 (Dwyer et al., 2006; Merritt et al., 2006). Consistent with the relatively large binding surface between CXCR1/2 and their chemokine ligands, studies have found that repertaxin, SB265610, SCH527123, and related analogs act as allo-
steric inhibitors (Bertini et al., 2004; Casilli et al., 2005; Garau et al., 2006; Gonsiorek et al., 2007; Moriconi et al., 2007; Nicholls et al., 2008; Bradley et al., 2009; de Kruijff et al., 2009).

Our CXCR1/2 antagonist discovery efforts have focused on refinement and pharmacological characterization of the nicotinamide glycolate pharmaco-
phore (Fig. 1). Although a representa-
tive nicotinamide glycolate methyl ester 1 of this class was shown to potently antagonize CXCL1-mediated chemotaxis of primary human PMNs in vitro, with an IC50 of 42 nM (Cutshall et al., 2002), pharmacological mechanisms were not evaluated. Through the use of tritiated derivatives and direct electroporation of compounds into PMNs, we show that the methyl ester form (e.g., 3) permeates through the PMN cell membrane, is rapidly hydrolyzed to the nicot-

amide glycolate acid (e.g., 4), and then accumulates intracel-
ularly to millimolar concentrations, where it acts as a
CXCR2 antagonist. The accumulated acid is subject to ef-

ux from the cell by a probenecid-sensitive transporter, probably an organic anion transporter (OAT). The studies presented herein thus describe the pharmacology of cer-
tain hydrolytically sensitive forms of this pharmaco-
phore class and demonstrate that the nicotinamide glycolates require intracellular access to antagonize CXCR2 signaling. The implications of these findings are discussed with regard to developing future related structural classes of compounds based on this pharmaco-
phore, which may be suitable for therapeutic implementation.

Materials and Methods

Chemistry. All chemicals and reagents for synthesis were pur-
chased from Aldrich Chemical (Milwaukee, WI), and solvents were purchased from VWR International (West Chester, PA). All reagents and solvents were used as received. Compounds 1 to 4 were prepared as described by Cutshall et al. (2002), and synthetic methods and characterization data. To prepare the tritiated derivatives of 3 and 4 (Fig. 2), 5,6-dicloro-nicotinic acid was coupled with 4-fluoronicine using 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline to yield 7 in 72% yield. Compound 7 was then treated with methyl glycolate and sodium hydride to form the glycolate methyl ester 8 in 55% yield. The methyl ester was saponified to yield the corresponding acid 9 in quantitative yield through the use of aqueous LiOH in MeOH. The choro-substituted ester and acid were then treated with tritium gas over a heterogeneous catalyst (Moravek Biochemicals, Brea, CA).

![Fig. 1](attachment:Fig_1.png)  
**Fig. 1.** Selected members of the nicotinamide glycolate pharmaco-
phore class.

![Fig. 2](attachment:Fig_2.png)  
**Fig. 2.** Schematic showing the synthesis of [3H]3 and [3H]4. i) 2-
Ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline, 4-fluoronicine in di-
methyl formamide (72%); ii) NaH dispersion in mineral oil, methyl glycolate in tetrahydrofuran (55%); iii) [3H]2 (g), 10% palladium on activated carbon in ethanol; iv) 1 N LiOH in MeOH (100%). See supplemental data for detailed experimental procedures and charac-
terization data.
were monitored (concentration of CXCL1 was 25 nM), and changes in fluorescence (control) were added again at the same concentrations that were HBSS containing 10 mM HEPES. The test compounds or DMSO loading, the samples were centrifuged at 3000 g for both compound and control) for 30 min at 37°C. After dye efflux, the samples were centrifuged at 3000 g for 1 min, the supernatant of intracellular radioactivity internalized in the cell pellet was quantitated by scintillation counting (LS-6500 liquid scintillation counter; Beckman Coulter, Fullerton, CA) and correlated with a calibration curve for each tritiated derivative. Efflux studies were performed by loading PMNs (6 × 10⁷ cells in 8 ml of HBSS) with the carboxylic acid [³H]4 by a 20-min incubation with 20 μCi of the methyl ester [³H]3, followed by washing and then resuspending the cells in 20 ml of HBSS. Aliquots from this cell suspension were then taken at 5, 15, 30, 60, and 90 min and centrifuged. The supernatant was analyzed by reverse-phase HPLC using a Class Vp system (Shimadzu, Kyoto, Japan) with a Jupiter C18 column (5 μm, 300 Å, 25 × 0.46 cm) (Phenomenex, Torrance, CA) and eluted with acetonitrile/water [60%/40% (v/v)] containing 0.1% (v/v) trifluoroacetic acid at a flow rate of 1 ml/min. The amount of [³H]4 and [³H]3 was quantitated by scintillation counting and integrating peak radioactivity (see method for detecting conversion of [³H]3 to [³H]4 below). For studies that examined the effect of probenecid on efflux, cells were loaded as described above, except that cells were resuspended in 25 ml of HBSS with and without 2 mM probenecid (p-(dipropylsulfamoyl)benzoic acid). Probenecid HCl is acidic and was buffered with NaOH and then diluted in HBSS before use to exclude any effect due to pH on efflux.

Intracellular Conversion of [³H]3 to [³H]4. PMNs (6 × 10⁷ cells in 8 ml of HBSS) were incubated with 5 μCi of [³H]3, washed, and then lysed with 100 μl of 0.1% Triton X-100 at 0, 5, and 20 min, and the lysate was subjected to reverse-phase HPLC and scintillation counting, as described above. Radioactivity in peaks with retention times corresponding to 3 and 4 was integrated to determine the relative amounts of intracellular [³H]3 and [³H]4, respectively.

Calcium Flux Assay. PMNs (3.1 × 10⁷ cells in 1.7 ml) were suspended in HBSS without Ca²⁺ and Mg²⁺ (‘HBSS-’ containing 10 mM HEPES and Fura-2 acetoxymethyl ester dye (final concentration, 2 μg/ml). Cells were measured (in aliquots) and treated with the indicated compounds or control DMSO (1% DMSO final concentration for both compound and control) for 30 min at 37°C. After dye loading, the samples were centrifuged at 3000g for 1 min, the supernatants were removed, and the cell pellets were resuspended in HBSS containing 10 mM HEPES. The test compounds or DMSO (control) were added again at the same concentrations that were used during cell loading, and the cells were measured (in aliquots) into a 96-well microtiter plate (10⁶ cells/well). After 15 s of reading the basal level of fluorescence, CXCL1 or HBSS was added (final concentration of CXCL1 was 25 nM), and changes in fluorescence were monitored (λex, 340 nm; λem, 380 nm) every 5 s for 240 to 500 s at room temperature using a Fluoroscan Ascent FL microplate reader (Thermo Fisher Scientific, Waltham, MA). Maximal change in the ratio of fluorescence values at excitation wavelengths of 340 and 380 nm, expressed in arbitrary units over baseline (max-min), was used to determine the response. The effect of each compound on the CXCL1 response was normalized and expressed as a percentage of the DMSO control, which was designated as “100% response.” Curve fitting and calculation of the compound inhibitory concentration that reduced the level of the CXCL1 response by 50% (IC₅₀), or the compound agonist concentration that increases the level of the calcium release by 50% of the maximal agonist-induced change (EC₅₀) were determined by nonlinear regression analysis of the dose-response curves generated using Prism 4 (GraphPad Software, Inc., San Diego, CA).

Human Neutrophil Electroporation. PMNs were electroporated on ice using two discharges of a 25-μF capacitor at 1.75 to 2.5 kV/cm with a Gene Pulser II (Bio-Rad Laboratories, Hercules, CA) as described previously (DeLeo et al., 1996). Analysis of aliquots of the cells for trypan blue exclusion indicated 96 to 98% of the cells were permeabilized. Permeabilized cells were transferred to six-well tissue culture plates containing RPMI 1640 medium/10% fetal bovine serum without (no compound control) and with the indicated concentrations of test compound 2 (no DMSO was added for test compound or control, because the acid 2 was soluble in aqueous solution without requiring DMSO). After a 30-min incubation at 37°C and 5% CO₂, the cells were collected, washed, and resuspended in HBSS containing 0.1% bovine serum albumin. Analysis of aliquots of the cells for trypan blue exclusion indicated 97 to 98% of the cells recovered and excluded trypan blue.

De-Esterification in Human Plasma. A 1 mM stock solution of the test compound was prepared in DMSO and serially diluted to a concentration of 10 μM in phosphate-buffered saline. A 100-μl aliquot of the 10 μM test solution was added to 900 μl of previously frozen pooled human plasma (Innovative Research, Novi, MI) to yield a 1 μM final concentration with 0.1% DMSO. The plasma samples were then incubated at 37°C, and 100-μl aliquots were removed at time points 0, 15, 30, 60, 120, and 240 min, and diluted with 300 μl of acetonitrile containing 1 μM internal standard. The precipitated proteins were centrifuged, and the supernatant was decanted and analyzed by liquid chromatography-tandem mass spectrometry using a Micromass Quattro II mass spectrometer (Waters, Milford, MA) connected to a 10AD HPLC system (Shimadzu) using separation conditions identical to those used for evaluation of product purity (Supplemental Data). Multiple reaction monitoring mode was used for detection of esters 1, 5, and 6, and the internal standard. The first quadrupole was set to transmit the precursor ions MH⁺ at m/z 320.9 for 1, 396.9 for 5, 363.1 for 6, and 473.9 for the internal standard. The product ion spectra were monitored in the third quadrupole at m/z 109.8 for both esters 1 and 6, 90.8 for 5, and 134.9 for the internal standard. The product ion peaks were integrated, and normalized to the internal standard product ion peak.

Results

Pharmacologic Parameters of Nicotinamide Glycolates 1 to 4. Pharmacologic parameters in the CXCR2 signaling pathway were defined for compounds 1 to 4 (Table 1). The nicotinamide glycolate methyl ester 1 was a potent antagonist in whole-cell assays of CXCL1-mediated chemotaxis.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CXCL1 Chemotaxis IC₅₀</th>
<th>CXCL1 Ca²⁺ Flux IC₅₀</th>
<th>125I-CXCL8 Binding IC₅₀</th>
<th>CXCL1 [³H]GTP[γS] IC₅₀</th>
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<tr>
<td></td>
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<td>3</td>
<td>110</td>
<td>68</td>
<td>&gt;10,000d</td>
<td>N.D.</td>
</tr>
<tr>
<td>4</td>
<td>&gt;20,000</td>
<td>&gt;5000</td>
<td>N.D.</td>
<td>N.D.</td>
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</table>

N.D., not determined.

a Values as reported in Cutshall et al. (2002).

b PMNs.

c Membrane preparations from CHO cells stably expressing CXCR2.

d Same for 125I-CXCL1 binding (MDS Pharma Services, Bothell, WA).

TABLE 1 Pharmacologic parameters of the nicotinamide glycolates 1 to 4 Values are the geometric mean of two or more determinations.
(IC_{50} = 42 nM) and calcium flux (IC_{50} = 48 nM) in PMNs, but surprisingly it exhibited no antagonism in cell-free CXCR2 assays of either ^{125}I-CXCL8 binding, ^{125}I-CXCL1 binding, or CXCL1-stimulated[^{35}S]GTPyS exchange (Table 1). Conversely, the corresponding nicotinamide glycolate carboxylic acid 2 lacked activity in whole-cell assays of chemotaxis and calcium flux in PMNs but inhibited both radiolabeled CXCL8 binding (IC_{50} = 1.2 μM) and CXCL1-stimulated[^{35}S]GTPyS exchange (IC_{50} = 0.61 μM) in CXCR2 membranes. The equipotent but oxygen-containing nicotinamide glycolates 3 and 4 exhibited a similar activity profile in cell-free assays of ^{125}I-CXCL8 and ^{125}I-CXCL1 binding to CXCR2 membranes, and in whole-cell assays of CXCL1-mediated chemotaxis and calcium flux. Absent antagonism at N-formyl peptide receptors CCR1, CCR2, CCR3, and CCR9 supported the CXCR2-selectivity of compounds 1 to 4 (Cutshall et al., 2002). The structure-activity relationship of 1 and 2 in cell-free assays indicated that methyl esterification of the carboxylic acid was a sufficient perturbation to render 2 inactive at CXCR2. The correlation between negative charge and absent activity of the nicotinamide glycolates suggested that the cell membrane acted as a barrier to the passive diffusion of the carboxylic acid 2, which was otherwise active in cell-free assays. Based on these observations, we postulated that the uncharged and inactive methyl ester 3 accesses the intracellular space by passive diffusion, where it is deesterified to form the active carboxylic acid 2 that then binds an intracellular site and antagonizes CXCR2.

Uptake of Methyl Ester[^{3}H]3 and Carboxylic Acid[^{4}H]4. To quantify potential differences in the rate and magnitude of nicotinamide glycolate uptake between the acid and ester forms, PMNs were incubated over 90 min with varying concentrations of ester[^{3}H]3 or acid[^{4}H]4. Aliquots were removed at various times, and the amount of tritiated compound internalized was quantified by scintillation counting (Fig. 3). For ester[^{3}H]3, the amount of radioactivity internalized dramatically increased as a function of extracellular concentration and time, reaching intracellular concentrations 100- to 1000-fold greater than the extracellular concentration. At the highest extracellular concentration tested, incubation of PMNs in 10 μM[^{3}H]3 for 90 min resulted in the internalized radioactivity reaching an intracellular concentration of ~3 mM based on a mean PMN volume of 334 fL (Nibbering et al., 1990). In contrast, the uptake of acid[^{4}H]4 was very limited, with intracellular accumulation detected only at the highest concentration tested. These data confirmed that the methyl ester was readily internalized into the PMN intracellular space, but the corresponding acid was substantially excluded by the cellular membrane, which is consistent with intracellular access being vital to activity.

Intracellular De-Esterification of Methyl Ester[^{3}H]3 to Carboxylic Acid[^{4}H]4. The absence of antagonism of the methyl ester 1 in cell-free assays, together with vigorous cellular uptake and potent nanomolar antagonism of the esters in whole PMNs, led us to hypothesize that ester antagonism of CXCR2 signaling required conversion of the methyl ester to the corresponding acid within the PMN. To test this hypothesis, PMNs were incubated with[^{3}H]3 and the PMNs were then collected at various times over 60 min and lysed. The lysates were then analyzed to quantify the relative amounts of the methyl ester[^{3}H]3 and the corresponding acid[^{4}H]4 (Fig. 4). After 5-min incubation, virtually

Fig. 3. Uptake of[^{3}H]3 (top) and[^{4}H]4 (bottom) in PMNs. After incubation of PMNs with the indicated concentrations of[^{3}H]3 or[^{4}H]4, aliquots were washed and centrifuged at the indicated times. The molar amount of radioactivity internalized was determined by measuring the radioactivity in the cell pellet by scintillation counting and correlating this to a calibration curve prepared separately with[^{3}H]3 and[^{4}H]4 (data not shown). Data show the mean molar amount of internalized radioactivity from triplicate determinations.

Fig. 4. Representative chromatogram demonstrating separation of a comixture of the ester[^{3}H]3 from the acid[^{4}H]4 (A). Intracellular conversion of[^{3}H]3 to[^{4}H]4 (B). PMNs were incubated with 5 μCi of[^{3}H]3, washed, and lysed at the indicated times, and the lysates were subjected to reverse-phase HPLC and scintillation counting. Radioactivity in peaks with retention times corresponding to 3 and 4 were integrated to determine the relative amounts of intracellular[^{3}H]3 and[^{4}H]4, respectively. Data show the mean intracellular radioactivity of each compound from triplicate determinations.
all radioactivity (>96%) detected in the PMN lysate corresponded to the hydrolyzed acid form. There was no de-esterification of the methyl ester \(^{[3}H\)3 in control experiments that subjected \(^{[3}H\)3 to the same incubation and lysis conditions in the absence of cells (data not shown). These data indicate that the nicotinamide glycolate esters are rapidly converted intracellularly to the corresponding acid, and in combination with their absent activity in cell-free assays, suggest that the acid is the active intracellular antagonist.

**Antagonism and Susceptibility to De-Esterification.** The esterase susceptibilities of the nicotinamide glycolates were determined by incubation in previously frozen human plasma. Whereas the nicotinamide glycolate methyl ester 1 and benzyl ester 5 were rapidly de-esterified to the corresponding acid 2, the sterically hindered tert-buty1 ester 6 exhibited complete resistance to ester hydrolysis out to 240 min (Fig. 5). Susceptibility to de-esterification was found to correlate with activity of the ester precursor in antagonizing CXCL1-stimulated calcium flux in whole PMNs (Table 2), consistent with the liberated acid 2 being the active antagonist in the intracellular compartment. The 240-fold greater potency of benzyl ester 5 compared with methyl ester 1 was probably the result of its greater lipophilicity, as suggested by the calculated octanol/water partition coefficients (Table 2).

**Electroporation of Carboxylic Acid 2 into PMNs.** To directly evaluate the possibility that carboxylic acid 2 is the active antagonist that acts intracellularly, 2 was provided access to the intracellular compartment of PMNs through electroporation (Fig. 6). Whereas compound 2 in the absence of electroporation was unable to inhibit CXCL1-mediated calcium release in PMNs at an extracellular concentration up to 5 \(\mu\)M, electroporation of 2 enabled potent and significant inhibition of CXCL1-mediated intracellular calcium release at extracellular concentrations of 0.1, 0.5, and 1.0 \(\mu\)M (IC\(_{50}\) = 260 ± 30 nM; ***, \(p < 0.01\) relative to the untreated control, one-way ANOVA, Dunnett’s multiple comparison test). This was not an artifact of electroporation, because parallel controls lacking antagonist retained chemotactic and intracellular calcium release responses upon treatment with CXCL1. Trypan blue exclusion controls confirmed that PMN membranes had completely resealed at the time antagonism was measured. These data show that intracellular nicotinamide glycolate acid 2 alone is sufficient to affect potent CXCR2 antagonism in PMNs. This observation together with its rapid and nearly quantitative liberation from the methyl ester 1 strongly implicates the ester as an inactive precursor that gives rise to the corresponding acid 2 as the active intracellular antagonist.

**Efflux of Carboxylic Acid \(^{[3}H\)4 from PMNs.** The absence of antagonism of the carboxylic acid form of the nicotinamide glycolates in whole-PMN assays suggested that the cell membrane may be acting as a bidirectional barrier to diffusion that could potentially trap the active carboxylic acid liberated from the ester precursor inside the cell. To examine the ability of PMNs to efflux the carboxylic acid form of the nicotinamide glycolate, PMNs were loaded with the carboxylic acid \(^{[3}H\)4 by incubation with the methyl ester \(^{[3}H\)3 precursor (Fig. 7). At 30, 60, and 90 min, there was a significant accumulation of radioactivity in the supernatant compared with the amount in the supernatant at 5 min (***, \(p < 0.01\), one-way ANOVA, Dunnett’s multiple comparison test). All the radioactivity was accounted for by the carboxylic acid \(^{[3}H\)4, with no methyl ester \(^{[3}H\)3 precursor detected. These observations suggest that the cell membrane did not irreversibly trap the active carboxylic acid within the cell. Instead, the data are consistent with efflux of the nicotinamide glycolate carboxylic acid from the PMN.

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**Table 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Ester R Group</th>
<th>CXCL1 Ca(^{2+}) Flux IC(_{50})</th>
<th>Plasma Half-Life*</th>
<th>Log P*</th>
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<td>min</td>
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<tr>
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<td>tert-Butyl</td>
<td>&gt;10,000</td>
<td>&gt;240</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* Esterase activity in human plasma at 37°C using a compound concentration of 1 \(\mu\)M.

* Octanol:water partition coefficient calculated using the Molinspiration property engine version 2009.01 according to the mILogP2.2 method (http://www.molinspiration.com).
The anionic nature of carboxylic acid [\(^{3}\)H]4 suggested that the observed efflux might be mediated by an OAT. To explore this possibility, we examined the effect of the OAT inhibitor probenecid on efflux of [\(^{3}\)H]4 from PMNs. PMNs were loaded with the carboxylic acid [\(^{3}\)H]4 and exposed to probenecid (Fig. 8). Compared with untreated controls, probenecid significantly inhibited [\(^{3}\)H]4 efflux from PMNs, as measured by its accumulation in the supernatant (***, *p < 0.001, two-way ANOVA). Probenecid also significantly inhibited efflux, as measured by [\(^{3}\)H]4 loss from the cell pellet, and after 90 min there was ~2.5-fold more radioactivity in the probenecid-treated PMN pellets than in the untreated control pellets. These data suggest that a PMN OAT is responsible at least in part for efflux of the active nicotinamide glycolate carboxylic acid from the intracellular PMN compartment.

**Kinetics of CXCR2 Inhibition in PMNs after Methyl Ester 1 Washout.** The observed efflux of the active nicotinamide glycolate acid from PMNs led us to consider how efflux would affect the kinetics of CXCR2 inhibition after washout of the methyl ester 1 from the extracellular space (Fig. 9). To examine this further, PMNs were incubated in the presence or absence of 1 \(\mu\)M methyl ester 1 for 30 min, loaded with the cell-permeable calcium dye Fura-2, and then thoroughly washed and resuspended in assay buffer. The intracellular CXCL1-stimulated calcium flux was measured at times ranging from 0 to 3.5 h. To control for changes to intracellular calcium related to PMN viability over the course of the experiment, percentage of calcium flux is expressed relative to the dynamic range established by a positive and negative control at each time point. Compared with untreated PMNs, calcium flux was inhibited in PMNs treated with 1, and this inhibition was sustained throughout the duration of the experiment (3.5 h).

**Discussion**

CXCR2 has attracted considerable attention as a potential drug target because of its involvement in different inflammatory diseases, particularly chronic pulmonary inflammatory diseases such as chronic obstructive pulmonary disease, where increased numbers of neutrophils in the sputum of patients has been shown to correlate with increased levels of IL-8 (Keatings et al., 1996; Chapman et al., 2009). As a consequence, different classes of small-molecule CXCR2 antagonists have been developed that seem to share a common mechanism of allostERIC, rather than competitive inhibition (Bertini et al., 2004; Casilli et al., 2005; Garau et al., 2006; Gonsiorek et al., 2007; Moriconi et al., 2007; Nicholls et al., 2008; Bradley et al., 2009; de Kruijf et al., 2009). Allosteric inhibition is likely to be a common mechanism for drug leads arising from small-molecule screening efforts against chemo-
kine receptors, given the large extracellular ligand-receptor interface that a competitive inhibitor must confront. Allosteric inhibition has thus been observed for small-molecules targeting other chemokine receptors, such as AMD3100 at CXCR4, TAK-779 at CCR5, and BX 471 at CCR1 (Allen et al., 2007).

For CXCR1/2, studies have found that the antagonists repertaxin, SB265610, SCH527123, and their related analogs act as allosteric inhibitors. Whereas natural ligands such as CXCL1/8 bind to the extracellular orthosteric binding site at the N terminus and extraacellular loops of CXCR1/2 (Allen et al., 2007), small-molecule antagonists seem to bind to allosteric sites at the seven-transmembrane domain, as demonstrated for repertaxin at CXCR1 (Bertini et al., 2004; Allegretti et al., 2005), or to a putative intracellular site of the CXCR2 receptor, as shown for the diarylurea SB332235 (Nicholls et al., 2008). Studies using radiolabeled SB265610 demonstrated competition between this member of the diarylurea class of antagonists and the structurally unrelated thiazolopyrimidine class (Walters et al., 2008), suggesting that both classes share a common binding site (de Kruijf et al., 2009). Conversely, radiolabeled SB265610 failed to compete with structurally unrelated imidazolylpyrimidine antagonists (Ho et al., 2006; de Kruijf et al., 2009), supporting the notion that distinct allosteric antagonist sites exist on CXCR2 (Ho et al., 2006; de Kruijf et al., 2009). Although data are therefore available on the binding sites of some CXCR2 antagonists, more research is required to explore and define other target sites suitable for therapeutic development of CXCR2 antagonists.

Our CXCR2 inhibitor discovery program has focused on the nicotinamide glycolate pharmacophore. The data presented in this study support a mechanism of action for the carboxylic acid form of the pharmacophore at an intracellular site. Candidates for this site include 1) an allosteric intracellular site on CXCR2, as suggested for the diarylurea class of antagonists (Nicholls et al., 2008); 2) a site on an intracellular CXCR2-associated protein; and/or 3) a site formed as a result of a ternary complex between CXCR2 and another protein.

The nicotinamide glycolate ester exhibited no antagonist activity in cell-free assays, but the tritiated derivative was rapidly internalized by PMNs and de-esterified to the carboxylic acid in the cell interior, consistent with a model in which the ester serves primarily as a lipophilic precursor that efficiently translocates into the intracellular space to liberate the active carboxylic acid. Indeed, the rates of de-esterification and passive diffusion (estimated by calculated log P values) in a series of related esters positively correlated with ester potency in antagonizing CXCL1-stimulated calcium flux in whole PMNs, supporting the pivotal role intracellular activation plays in the inhibitory mechanism of this class of compounds. Efflux of the active acid from PMNs was inhibited by probenecid, consistent the involvement of an organic acid transporter. Whereas up to 5 μM of the nicotinamide glycolate acid was inactive in whole-cell assays, the same compound inhibited calcium flux with an IC₅₀ of 260 ± 30 nM after PMNs were electroporated. Thus, direct evidence supporting the nicotinamide glycolate acid as the active intracellular antagonist was obtained using electroporation to directly load PMNs.

For studies involving radiolabeled nicotinamide glycolates, tritiated derivatives were prepared only for the nicotinamide glycolates, where X = 0 (i.e., 3 and 4), because the sulfur atom in derivatives 1 and 2 would have been incompatible with the palladium catalyst used in the tritiation reaction. Radiolabeled nicotinamide glycolate acid or ester did not bind to CXCR2 in Chinese hamster ovary (CHO) cell membranes at up to 5 μM (data not shown). The reason for the absence of binding in membrane preparations is unclear. One possibility consistent with the observed inhibition of CXCL1-stimulated [³⁵S]GTP-S exchange by the nicotinamide glycolate acid is that a ternary complex of chemokine (CXCL1 or CXCL8) with CXCR2 may be required to place the receptor in a conformation permissive to nicotinamide glycolate binding. The absence of other required membrane-associated proteins in the CHO cell membranes or their loss during the membrane preparation process are alternative possibilities. Studies to explore these possibilities are underway.

Treatment of PMNs with the nicotinamide glycolate ester resulted in a dramatic intracellular accumulation of the active acid to levels 100- to 1000-fold greater than the extracellular ester concentration and in some cases reached millimolar concentrations. The concentrative accumulation of the acid indicated that the rate of ester uptake and intracellular de-esterification are together more rapid than the rate of efflux of the active carboxylic acid. Given the extent of accumulation, it is perhaps not surprising that CXCR2 inhibition, as measured by inhibition of calcium flux in PMNs, was sustained for at least 3.5 h after washout of the ester from the extracellular space. For example, extrapolation of the loading and efflux kinetics of the tritiated ester derivative predicts an intracellular concentration of 60 μM for the active carboxylic acid after 1.5 h of washout, well above the sub- to low-micromolar potency observed for the carboxylic acid in cell-free assays and electroporated PMNs. Taken together, these data reconcile differences observed between the activity of the esters (methyl or benzyl) and the corresponding acid in electroporated PMNs. Although the esters exhibited potent nanomolar (methyl ester) to subnanomolar (benzyl ester) activity in whole PMNs, the acid had weaker activity in electroporated PMNs. Due to the concentrative mechanism involved, the active acid was presumably able to attain the requisite intracellular concentration to antagonize CXCR2 from lower extracellular concentrations of the ester precursor.

As extensively reviewed by Beaumont et al. (2003), ester prodrugs are most often used to enhance the lipophilicity and, thus, the passive membrane permeability of water-soluble drugs through the intestinal barrier by masking charged groups such as carboxylic acids and phosphates. Once in the body, the ester bond is hydrolyzed to release the active principle into the circulation by ubiquitous esterases found in the intestine, blood, liver, and other tissues, including carboxyl esterases, acetylcholinesterases, butyrylcholinesterases, paraoxonases, and arylesterases. The major aim of the prodrug approach is to quantitatively produce high concentrations of the active principle in the systemic circulation, not to mediate the entry of the prodrug into the target cell where the active principle would otherwise be unable to enter. The coupling of a cell-permeable but inactive ester precursor to intracellular liberation of the active antagonist in the target cell is thus an unusual delivery mechanism among ester prodrugs, with the lactone forms of HMG-CoA reductase inhibitors (e.g., lovastatin and simvastatin) that
target hepatocytes and the CXCR2 antagonists that target PMNs reported herein being examples.

To our knowledge, the nicotinamide glycolate esters are the only reported CXCR2 antagonists that require intracellular activation, as reported here. The coupling of a cell-permeable but inactive ester precursor to intracellular activation of the active antagonist is therefore unique among CXCR2 antagonists. This mechanism placed a challenging constraint on identifying ester congeners suitable for therapeutic implementation that required identifying a balance between the kinetics of intracellular activation and extracellular inactivation (i.e., in plasma), where both processes are mediated by de-esterification of the precursor. Cellular potency and instability in the plasma were thus invariably coupled to one another. The potential for rapid first-pass clearance by esterase activity in the intestine and liver were additional concerns. Ester forms that were highly potent in vitro necessarily exhibited rapid inactivation kinetics that precluded their usage in vivo. These features led us to abandon the nicotinamide glycolate ester pharmacophore for further development. However, the mechanism of ester activation elucidated herein gave us insight to strategies for developing new nicotinamide pharmacophore classes with therapeutic potential. One strategy we have pursued successfully is to identify new nonester nicotinamide pharmacophore classes that are cell permeable and potent in the intracellular space but stable in the plasma. Our developments and in vivo results related to these new classes of nicotinamide antagonists will appear in subsequent reports.

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References


**General Synthesis and Characterization Methods**

Synthetic reaction progression was monitored by TLC using precoated aluminum backed silica gel plates with fluorescent indicator (EMD Chemicals, Gibbstown, NJ). $^1$H NMR (300.1 MHz) spectra were recorded on a Bruker AV300 spectrometer. MS spectra were recorded on a Micromass Quattro II mass spectrometer (Waters, Milford, MA), operating under positive ion mode using an electrospray ionization probe, connected to a Shimadzu 10AD HPLC system (Shimadzu, Kyoto, Japan) equipped with a UV detector (SPD-10AVvp), two pumps (LC-10ADvp), an autosampler (SIL-HT), degasser (DGU-14A) and a column oven (CTO-10AC). Reverse-phase HPLC separations were performed with a Phenomenex Gemini C$_{18}$ column (2.0 x 50 mm; 110 Å, 5 μm) at 35 °C utilizing gradient elution (5 – 95% B over 12 min, solvent A = 0.1% formic acid/H$_2$O, solvent B = 0.1% formic acid/MeCN, flow rate = 0.2 ml/min, l = 254 nm). The purity of all compounds was determined to be ≥ 95% by reverse-phase HPLC.

**Synthesis of [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl]-acetic acid benzyl ester 5**

To a solution of $N$-(4-fluoro-phenyl)-6-mercapto-nicotinamide [0.25 g, 1 mmol, 1 eq. (as prepared by Cutshall et al. 2002)] in anhydrous dimethylformamide (2 ml), benzyl bromoacetate (0.16 ml, 1 mmol, 1 eq.) was added, followed by triethylamine (0.14 ml, 1 mmol, 1 eq.). A crystalline material immediately formed. After 1 hour at room temperature, the reaction mixture was diluted into ethyl acetate (50 ml) and the organic layer washed with water, 0.1 N aq. HCl, water, brine, and dried over Na$_2$SO$_4$. The reaction was filtered, and the solvent removed by rotary evaporation. The crude material was purified by silica gel column chromatography (ethyl acetate/hexanes, 1:2 as eluent) to yield 278 mg (70%) as an off white solid. TLC R$_f$ (ethyl acetate/hexanes, 1:1) = 0.60; HPLC R$_f$ = 11.21 min; [MH]$^+$ (ESI-MS) = 397.01; $^1$H NMR (300
MHz, d$_6$-DMSO) $\delta$ 4.19 (s, 9H), 5.16 (s, 2H), 7.21 (t, 2H), 7.34 (m, 5H), 7.53 (d, 1H), 7.78 (dd, 2H), 8.14 (dd, 1H), 8.88 (dd, 1H), 10.34 (s, 1H).

**Synthesis of [5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl] acetic acid tert-butyl ester 6**

To a solution of N-(4-fluoro-phenyl)-6-mercapto-nicotinamide [0.25 g, 1 mmol, 1 eq. (as prepared by Cutshall et al. 2002)] in anhydrous dimethylformamide (2 ml), tert-butyl bromoacetate (0.15 ml, 1 mmol, 1 eq.) was added, followed by triethylamine (0.14 ml, 1 mmol, 1 eq.). A crystalline material immediately formed. After 1 hour at room temperature, the reaction mixture was diluted into ethyl acetate (50 ml) and the organic layer washed with water, 10% aq. citric acid, water, brine, and dried over Na$_2$SO$_4$. The reaction was filtered, and the solvent removed by rotary evaporation. The crude material was purified by silica gel column chromatography (ethyl acetate/hexanes, 1:2 as eluent) to yield 199 mg (55%) as an off white solid. TLC $R_f$ (ethyl acetate/hexanes, 1:1) = 0.77; HPLC $R_t$ = 11.46 min; [MH]$^+$ (ESI-MS) = 307.06; $^1$H NMR (300 MHz, d$_6$-DMSO) $\delta$ 1.41 (s, 9H), 4.00 (s, 2H), 7.21 (t, 2H), 7.51 (d, 1H), 7.76 (dd, 2H), 8.15 (dd, 1H), 8.93 (s, 1H).

**Synthesis of 5,6-dichloro-N-(4-fluoro-phenyl)-nicotinamide 7**

To a solution of 5,6-dichloronicotinic acid (3.84 g, 20 mmol, 1 eq.) and 4-fluoroaniline (2.22 g, 20 mmol, 1 eq.) in dimethylformamide (60 ml) was added 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline (EEDQ, 4.94 g, 20 mmol, 1 eq.). The reaction solution was stirred at room temperature under nitrogen overnight. The reaction solution was then diluted into ethyl ether (100 ml) and washed with water, 0.1 N aq. HCl, water, 5% aq. NaHCO$_3$, water, brine, and dried over MgSO$_4$. The mixture was filtered, and the solvent was removed by rotary evaporation to
yield 4.087 g (72%) as an off white solid. TLC Rf (ethyl acetate/hexanes, 1:2) = 0.38; HPLC Rt = 10.78 min; [MH]+ (ESI-MS) = 286.81; 1H NMR (300 MHz, d6-DMSO) δ 7.24 (t, 2H), 7.76 (dd, 2H), 8.62 (d, 1H), 8.89 (d, 1H), 10.60 (s, 1H).

Synthesis of [3-chloro-5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl]-acetic acid methyl ester 8

To a solution of 5,6-dichloronicotinamide 7 (1.2 g, 4.2 mmol, 1 eq.) and methyl glycolate (0.65 ml, 8.4 mmol, 2 eq.) in anhydrous tetrahydrofuran (40 ml) was added sodium hydride (60% dispersion in mineral oil, 0.34 g, 8.4 mmol, 2 eq.) under argon at room temperature. Vigorous bubbling was seen, and the reaction mixture became a cloudy yellow color. After 3 hours, the reaction mixture was diluted into ethyl acetate (100 ml), and the combined organic layer was washed with water, 0.1 N aq. HCl, water, brine, and dried over Na2SO4. The mixture was filtered, and the solvent was removed by rotary evaporation to yield an oil. The oil was purified by silica gel column chromatography (ethyl acetate/hexanes, 1:3 as eluent) to yield 0.78 g (55%) as a white solid. TLC Rf (ethyl acetate/hexanes 1:2) = 0.43; HPLC Rt = 10.63 min; [MH]+ (ESI-MS) = 339.01; 1H NMR (300 MHz, d6-DMSO) δ 3.70 (s, 3H), 5.12 (s, 2H), 7.21 (t, 2H), 7.75 (dd, 2H), 8.44 (d, 1H), 8.64 (d, 1H), 10.38 (1H).

Synthesis of [3-chloro-5-(4-fluoro-phenylcarbamoyl)-pyridin-2-ylsulfanyl]-acetic acid 9

To a solution of methyl ester 8 (0.2 g, 0.64 mmol, 1 eq.) in methanol (15 ml) was added 1 N aq. LiOH (1.3 ml, 1.3 mmol, 2 eq.) at room temperature. After 2 hours, the reaction mixture was concentrated by rotary evaporator and diluted into 0.1 N HCl (15 ml) and cooled at 5 ºC overnight. The resulting precipitate was filtered, washed with water and dried to yield 185 mg (100%) as a white solid. TLC Rf (1% acetic acid/ethyl acetate) = 0.26; HPLC Rt = 9.83 min;
[MH]$^+$ (ESI-MS) = 325.10; $^1$H NMR (300 MHz, d$_4$-MeOH) $\delta$ 5.03 (s, 2H), 7.09 (t, 2H), 7.67 (dd, 2H), 8.32 (d, 1H), 8.61 (d, 1H).


The tritiation of 8 and 9 to afford $[^3]$H-3 and $[^3]$H-4, respectively, and subsequent purification were performed at Moravek Biochemicals (Brea, CA, USA). The tritiated products were purified by C$_{18}$ reverse phase preparative HPLC on a Thermo Finnigan P4000 system using 50% aq. EtOH with 0.1% TFA, $\lambda$ = 274 nm. The final purified $[^3]$H-3 and $[^3]$H-4 had radiochemical purities >99.7% with specific activities of 18 Ci/mmol.