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Full Papers

An Inhibitor of CCL2-Induced Chemotaxis from the Fungus *Leptoxyphium* sp.

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A biological screen used to identify inhibitors of monocyte chemotactic protein-1 (CCL2)-induced chemotaxis was applied in the activity-guided fractionation of an extract from a fungus of the genus *Leptoxyphium* sp. Inhibition of CCL2-induced chemotaxis was traced to a new dichlorinated diketopiperazine, cyclo(13,15-dichloro-L-Pro-L-Tyr). A structure−activity relationship (SAR) study evaluating relative activities of cyclo(13,15-dichloro-L-Pro-L-Tyr) and a nonchlorinated homologue cyclo(L-Pro-L-Tyr) showed that the dichlorinated molecule was 10- to 20-fold more active than the nonchlorinated form, while no activity was observed for cyclo(D-*N*-methylLeu-L-Trp).

Monocyte chemotactic protein-1 (CCL2, also known as MCP-1) has been implicated in both acute and chronic inflammatory and autoimmune diseases associated with infiltration of monocytes, macrophages, dendritic cells, NK cells, basophils, and memory T-cells.[1] CCL2 is a chemokine peptide ligand, 8.6 kDa containing 76 amino acid residues, which binds to and activates the seven transmembrane G-protein coupled receptor CCR2. Activation of chemokine receptors is central to tumor cell metastasis and promotion of angiogenesis. High levels of expression of CCR2 are associated with advanced stages of multiple myeloma. The formation of a chemokine/chemokine receptor complex initiates a cascade of cellular signals resulting in the activation of downstream kinases, lipases, and adhesion molecules, which generates a number of potential regulatory points for chemokine receptor function. As 7TM-GPCRs are the largest class of receptors, many approaches have previously been taken to devise small-molecule antagonists. Evaluation of small molecular weight chemokine receptor antagonists as anticancer agents is still in its infancy, with only limited numbers of studies to evaluate potential efficacy having been reported.[1]

An exploratory CCL2-induced monocyte migration assay screening of 80 randomly selected extracts of fungal fermentations obtained from the DTP Repository in Frederick, MD, uncovered one dichloromethane (DCM) extract showing strong inhibition of migration induction.[1,2] This extract was made from a culture of *Leptoxyphium* sp., also known as *Caldariomyces,* a genus that has been utilized for the production of the metalloenzyme chloroperoxidase, i.e., EC 1. 11. 1. 10,[3] useful in bioorganic synthesis.

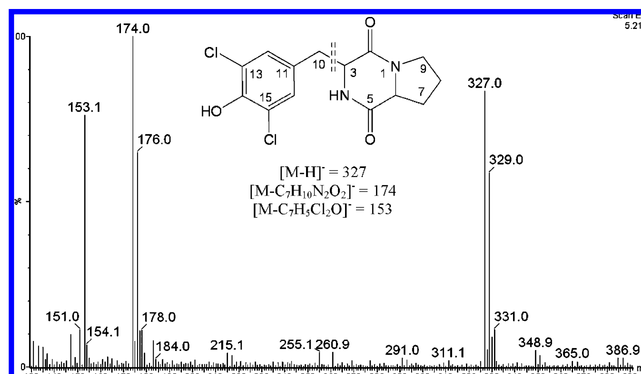

Figure 1. LRESIMS of **1** in MeOH. The fragmentation pattern clearly indicates the chlorination of tyrosine.

Bioactivity-guided fractionation was applied to isolate a CCL2-active substance (**1**) present in the active *Leptoxyphium* sp. extract, which, by mass spectrometric analysis, showed an isotopic pattern typical of a compound containing two chlorine atoms and with a molecular weight of 328 (Figure 1). The closely occurring UV

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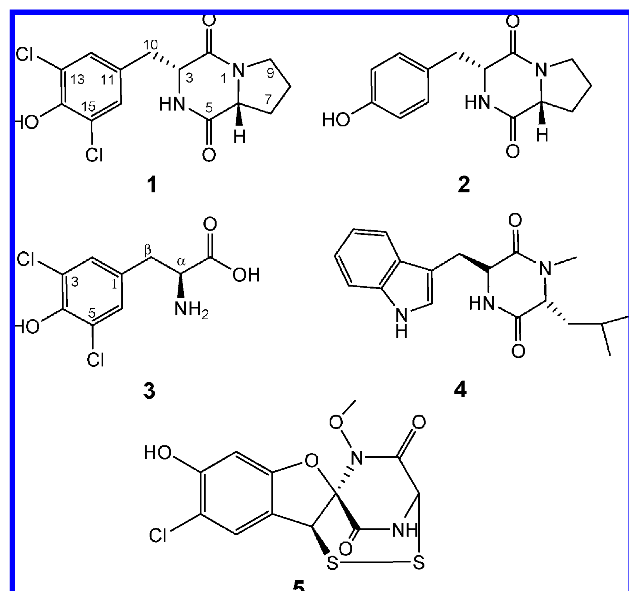
maxima at 283 and 289 nm were suggestive of an indole substructure or a *p*-substituted phenol. ^{1}H NMR experiments in methanol-d_4 and DMSO-d_6 revealed 12 nonexchangeable and two exchangeable protons. The presence of two amide groups (δ_C 166.6, 170.8) suggested that the molecule was of amino acid origin, which when incorporated into a microbial secondary metabolite of MW 328, was indicative of its formation from two residues. Three sets of adjacent methylene protons were evident in the HSQC/COSY data, signifying a proline (Pro) substructure. A ^{1}H NMR singlet at δ 7.1 integrating to two protons, which could not be resolved in methanol-d_4, DMSO-d_6, or acetone-d_6, suggested an element of symmetry within a conjugated ring. The six-membered ring of tyrosine (Tyr) provides an environment for the symmetrical positioning of the aromatic protons as well as two chlorine atoms. Chlorination at the C-13 and C-15 positions (δ 123, each) was suggested by the absence of an 8 Hz HMBC correlation with the H-10 methylene protons (δ 3.10, 2.97). Also, the presence of strong HMBC correlations of the C-12 and C-16 protonated carbons (δ 130.6, each) with the H-10 methylene protons further substantiated the assignment of the chlorine atoms. That a diketopiperazine structure was composed from the combination of a Pro residue and a dichlorinated Tyr was supported by a three-bond HMBC correlation of H-9 (from Pro) to C-2 (from Tyr). Low-resolution negative ion ESIMS performed at elevated cone voltage (70 V) yielded ions at m/z = 174 and 153 (Figure 1), attributed to fragmentation of the molecule at the C-3/C-10 position and providing further evidence for the proposed structure. The configuration of the amino acids of **1** could not be established by optical rotation[4,5] due to lack of material. Interestingly, comparison of literature coupling constants for the H-3/H-10a,b spin systems was not a definitive means of establishing configuration because the magnitude of the value depended on the solvent used. ^{1}H NMR spectra of **2**,[5] a commercially available nonchlorinated homologue of **1**, in methanol-d_4 yielded coupling constants of 5 Hz for both H-3/H-10a and H-3/H-10b, whereas in $CDCl_3$, coupling constants were not equivalent, with values of 10 and 3 Hz. Therefore, Marfey's method was applied to establish configuration.[6] Appropriate D- and L-Pro standards were readily available, but the modified amino acid 3,5-dichloroTyr, **3**, had to be synthesized. A mixture of D/L-Tyr and a pure sample of D-Tyr were chlorinated in high yield at the C-3 and C-5 positions by exposure to chlorine gas in methanolic solution. Hydrolysis of 25 μg of **1** followed by FDAA (Marfey's reagent) derivatization and HPLC analysis of the hydrolysis mixture, and the amino acid standards, led to the designation of L-Pro-L-Tyr.



Although chloroperoxidase from *Leptoxyphium* is known to halogenate Tyr,[3] the possibility that the dichlorinated metabolite **1** could have arisen as an artifact of the extraction process, where DCM was utilized, required testing. A 5 mg aliquot of **2** was dissolved in 2 mL of DCM/MeOH (9:1) and incubated at 40 °C for 3 h and also at room temperature for 72 h. A highly sensitive HPLC/MS analysis, in which selective negative ion monitoring for the m/z couplet of 327/329 was utilized, showed no detectable **1** at either time point for a 60 μg injection of product.

Several chlorinated metabolites, including caldariomycin and 2-(3,5-dichloro-4-hydroxyphenyl)ethanol, have previously been isolated from live cultures of *Leptoxyphium*.[7] Dichlorinated Tyr-containing metabolites from other microorganisms include the cryptophycins,[8] from *Nostoc* sp., and the chloropeptins,[9] from *Streptomyces lavendulae*. Dichlorinated diketopiperazines have been reported from *S. griseoluteus*[10] and *Aspergillus* sp.,[11] but with chlorine substituents located on different rings of the molecule. A diketopiperazine dichlorinated on the Tyr residue has not previously been reported. A compound showing a negative ion couplet of m/z 293/295 was detected in the fermentation broth of the *Leptoxyphium*, consistent with a monochlorinated **1**, but was not isolated due to the small amount.

After determination of the identity of the lead bioactive compound (**1**) from *Leptoxyphium* sp., additional diketopiperazines were obtained and tested for chemotaxis inhibition. Compound **2** (**1** lacking chlorine), known to possess phytotoxicity,[5] also showed activity in the CCL2 assay. The indole diketopiperazine **4** tested inactive,[12] and aspirochlorine, **5**,[12] an antifungal monochlorinated diketodithiopiperazine biosynthesized by *Aspergillus fumigatus* from glycine,[13] was cytotoxic to the monocytes. A recent review of the biological activity and synthesis of diketopiperazines lists numerous activities,[14] including their potential as antitumor agents.

The measurement of monocyte migration was performed by mixing **2** with CCL2 (**2** + CCL2) or with monocytes (**2**/CCL2) in a micro-Boyden chamber (Figure 2).[15] As can be seen in Figure 3A, the addition of 100 μM **2** to 100 ng/mL CCL2 (**2** + CCL2) inhibited 50% of specific migration. However, adding the same amount of **2** to the monocytes (**2**/CCL2) did not inhibit migration. Pretreating the monocytes with **2** for 30 min prior to introduction into the micro-Boyden chamber did not increase the inhibitory effect (Figure 3B). Comparing the dichlorinated diketopiperazine **1** to the nonchlorinated form **2** shows that **1** is 10- to 20-fold more effective at inhibiting CCL2-induced monocyte migration (Figure 4). Neither diketopiperazine impacted monocyte survival *in vitro* (data not shown). To explore the possibility of high avidity direct binding competition between these compounds and radio-labeled CCL2, equal molar and 100-fold molar excesses of either **1** or **2** were mixed with primary monocytes and radio-labeled CCL2. At these ratios of **1** or **2** to radio-labeled ligand, no reduction in ligand binding was observed, indicating that these compounds are not high-avidity competitors for ligand binding (data not shown). No cellular toxicity was observed when **1**, **2**, or **4** at 100 μM was in contact with human monocyte cultures for 24 h. Further investigation would be needed to determine the precise mechanism of action for this class of compound. These observations indicate that the dichlorinated diketopiperazine **1** isolated from *Leptoxyphium* sp. can attenuate the migration of human monocytes without significant leukocyte toxicity, suggesting that **1** is a lead structure for future development of anti-inflammatory compounds.

Experimental Section

General Experimental Procedures. UV spectra were acquired on a Lambda 20 UV/vis spectrophotometer (Perkin-Elmer). NMR spectra were recorded with a Varian 500 MHz INOVA spectrometer in methanol-d_4 or DMSO-d_6 with TMS as internal standard. HRESIMS was performed with a Waters LCT Premier time-of-flight mass spectrometer. HPLC-MS hardware consisted of a Waters 600 pump, a Micromass ZQ electrospray mass spectrometer (cone voltage = 30), a

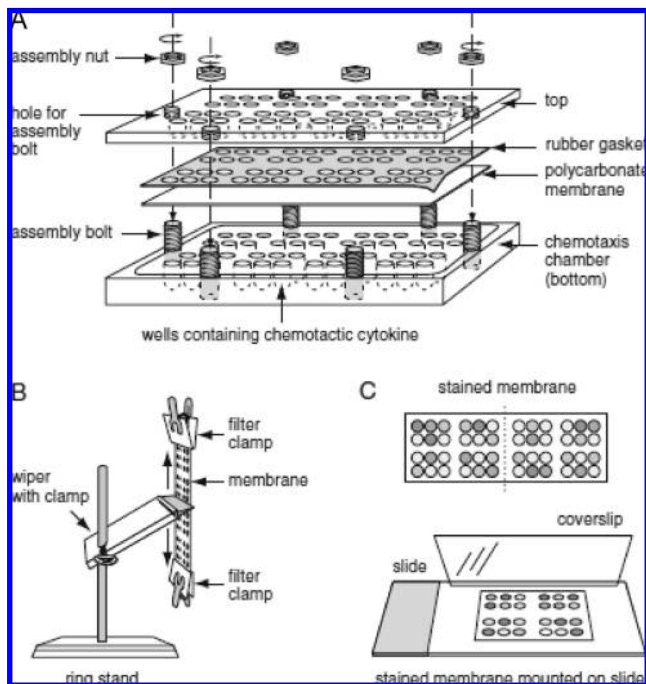


Figure 2. Experimental design of the monocyte migration assay. Monocytes were separated from CCL2 by a permeable filter membrane in a micro-Boyden chamber. Test compound **2** was added to CCL2 in the bottom of the chamber (**2** + CCL2) or to the monocytes in the top of the chamber (**2**/CCL2). After 1.5 h, the filter membrane was removed and stained with 3 step Dif-Quick stain. Monocyte migration was determined by counting the cells on the lower side of the membrane. Diagram from ref 15, reproduced with permission.

Waters 996 photodiode array spectrometer, and a Sedex 75 evaporative laser light scattering detector. Scale-up purification was accomplished on Sephadex LH-20 (Pharmacia) and by phenyl bonded-phase HPLC (Varian or Waters). Cyclo-L-Pro-L-Tyr (**2**) and standard D/L amino acids were obtained from Sigma-Aldrich.

Fermentation. *Leptoxyphium* sp. was isolated from fallen green fruit of *Gustavia superba* by R. Goos of the University of Rhode Island in 1995. A culture was deposited in the DTP Repository in Frederick, MD, as Q68C9963. Spores were grown on potato dextrose broth (PDB) agar for two weeks at ambient temperature. Mycelium from the agar were then added to 0.15 L of PDB broth and agitated at 200 rpm for one week at ambient temperature. Next, 15 L of soy-peptone-glucose-starch production medium was inoculated with the PDB seed culture and grown in ten 1.5 L aliquots in 6 L flasks for one week at 150 rpm agitation rate at room temperature. At harvest, the culture was colored black, the pH was 8, and glucose had been depleted. Production of **1** was consistently low in several media and with varying growth parameters. Attempts to improve the titer of **1** by addition of **2** and/or metal ions vanadium or copper, metals found in haloperoxidases, to fermentation media, were unsuccessful.

Extraction and Isolation. To whole fermentation broth was added a 10% volume of MeOH, which was high-shear homogenized, and extracted by partitioning against an equal volume of DCM. The DCM extract was rotary evaporated to dryness, yielding 0.5 g/L. Isolation of **1** from the DCM extract was accomplished by bioassay-guided fractionation with the CCL2 assay. The DCM solubles (14 g) were subjected to solvent partitioning with 350 mL each of acetonitrile (CH_3CN) and *n*-hexane. The CH_3CN-soluble material (7 g) was adsorbed to 60 g of C_{18} bonded-phase silica (Varian) and eluted under vacuum with an increasing percentage of CH_3CN in H_2O. The active material, 1.2 g (eluted at CH_3CN/H_2O (3:7)), was loaded onto a Sephadex LH-20 column (110 × 2.5 cm) and eluted with DCM/MeOH (1:3). The active fraction was next chromatographed by C_{18} HPLC with CH_3CN/20 mM NH_4OAc, pH 4.0, mobile phase and then through phenyl bonded-phase HPLC using the same solvent system. The highly enriched active compound was further purified using Sephadex LH-20 chromatography with MeOH as the mobile phase, yielding 0.4 mg of

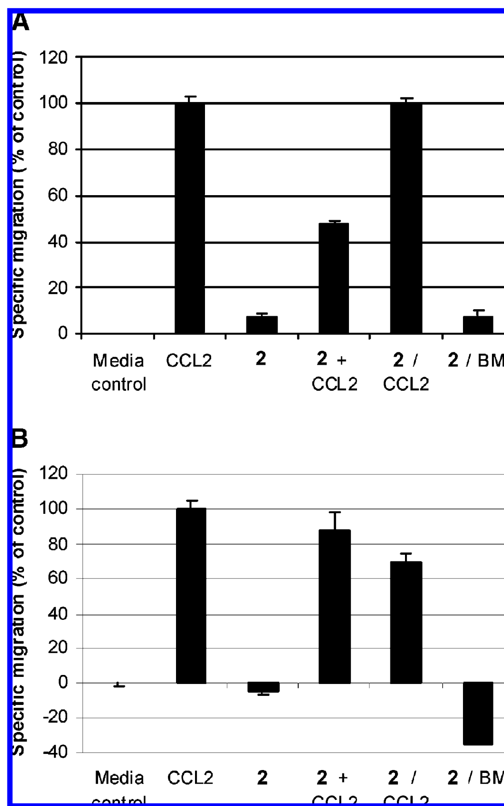


Figure 3. Diketopiperazines inhibit CCL2-induced monocyte migration. (A) Compound **2** blocks CCL2-induced migration. Compound **2** mixed with 100 ng/mL CCL2 (**2** + CCL2) in the lower micro-Boyden chamber inhibited 50% of CCL2 specific migration, while **2** mixed with the monocytes in the upper chamber (**2**/CCL2) did not. Compound **2** (100 μM) did not significantly alter monocyte migration either when placed in the lower wells alone (**2**) or when placed with the cells in the upper chamber over the media blank (**2**/BM). Concentration of **2** held constant at 100 μM. (B) 30 min pretreatment of monocytes with compound **2** does not increase the inhibitory effect. Test compound, monocyte, and CCL2 concentrations were the same as shown in part A. Monocytes were exposed to **2** for 30 min prior to adding to the micro-Boyden chamber.

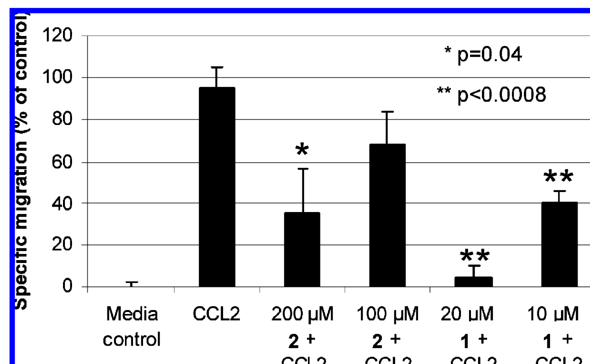


Figure 4. Dichlorinated diketopiperazine **1** is more potent than the nonchlorinated form **2**. Compound **1** (10 μM) was as efficient as 100 μM **2**. *p*-values were calculated using Prism-GraphPad 4 comparing migration at 100 ng/mL CCL2 to migration with **1** or **2** using a two-tailed Student's *t*-test. CCL2 was maintained at a constant concentration of 100 ng/mL.

1 at >95% purity as measured by ELSD, a yield of 0.003% from the DCM extract or 0.02 mg/L from the fermentation broth.

Cyclo(13,15-dichloro-L-Pro-L-Tyr) (1): white solid; UV (MeOH) λ_{max} (log ϵ) 283 (2.66), 289 (2.66), 310 (2.18) nm; ^{1}H NMR (methanol-

d_4, 500 MHz) δ 7.15 (2H, s, H-12 and H-16), 4.39 (1H, dd, $J = 5.0$, 1.5 Hz, H-3), 4.10 (1H, ddd, $J = 10.3$, 7.0, 2.0 Hz, H-6), 3.56 (1H, m, H-9a), 3.40 (1H, m, H-9b), 3.10 (1H, dd, $J = 14.0$, 5.0 Hz, H-10a), 2.97 (1H, dd, $J = 14.0$, 5.0 Hz, H-10b), 2.17 (1H, m, H-7a), 1.87 (2H, m, H-8a and H-8b), 1.40 (1H, m, H-7b); ^{13}C NMR (CD_3OD, 125 MHz) δ 170.8 (CO, C-5), 166.6 (CO, C-2), 151.2 (COH, C-14), 130.6 (CH, C-12), 130.6 (CH, C-16), 128.2 (C, C-11), 123.3 (CCl, C-13), 123.3 (C, C-15), 59.8 (CH, C-6), 57.3 (CH, C-3), 45.7 (CH_2, C-9), 36.3 (CH_2, C-10), 29.2 (CH_2, C-7), 22.5 (CH_2, C-8); LRESIMS (cone voltage = 70 V) *m/z* 327.0 $[M - H]^-$ (85), 174.0 $[M - C_7H_{10}N_2O_2]^-$ (100), 153.1 $[M - C_7H_5Cl_2O]^-$ (78); HRESIMS *m/z* $[M - H]^-$ 327.0287 (calcd for $C_{14}H_{13}Cl_2N_2O_3$, 327.0303).

Chlorination of Tyrosine. Chlorine gas was generated by the addition of 0.5 mL of concentrated HCl to 1.2 mL of household bleach in a septum-plugged test tube. The headspace above the mixture was sampled using a 20 mL syringe and injected into 2 mL of 10 mg/mL methanolic D/L-Tyr or D-Tyr (Sigma-Aldrich) solution. The addition of the chlorine gas and the subsequent reaction rapidly enabled solublization of the solid into CH_3OH. The high yield of the product precluded the need for purification prior to derivatization with FDAA.

3,5-Dichlorotyrosine (3). ^{1}H NMR (CD_3OD, 400 MHz): δ 7.21 (2H, s, H-3 and H-5), 4.29 (1H, dd, $J = 7.6$, 6.4 Hz, H-α), 3.17 (1H, dd, $J = 14.8$, 6.4 Hz, H-β), 3.03 (1H, dd, $J = 14.8$, 7.6 Hz, H-β).

Acid Hydrolysis and Determination of the Configuration of 1. [6] Compound **1** (25 μg) was dissolved in MeOH (100 μL) and transferred to a hydrolysis tube. The MeOH was removed under vacuum, and the tube was filled with 6 N HCl. After degassing, the solution was heated in an oil bath at 105 °C for 18 h, at which time the hydrolysis reaction was stopped. HCl was evaporated under a nitrogen stream, and the hydrolysis products were rinsed twice with MeOH followed by overnight drying under vacuum.

The hydrolysate of **1** and the amino acid standards D-Pro, D/L-Pro (Sigma-Aldrich), 3,5-dichloro-D-Tyr, and 3,5-dichloro-D/L-Tyr were dissolved in MeOH/H_2O (4:1, at 1 mg/mL) and transferred (25 μL) to separate HPLC autosampler vials. All traces of solvent were removed under vacuum. To each sample was added 15 μL of 6% TEA and 7.5 μL of FDAA (Marfey's reagent, Pierce). The samples were heated at 40 °C for 1 h and allowed to cool prior to C_{18} HPLC analysis with CH_3CN/5% aq HOAc (1:9 to 1:1).

CCL2-Induced Monocyte Migration Bioassay. Human monocytes were used to study the inhibition of CCL2-induced cell migration as previously described.[16,17] Fungal extracts and fractions were diluted in DMSO at a concentration of 100 mM and titrated as needed to determine IC_{50}. CCL2 (Peprotech. Rocky Hill, NJ) at 100 ng/mL in binding media (RPMI-1640 supplemented with 1% bovine serum albumin and 25 mM HEPES pH 8.0) was placed in the lower wells of a micro-Boyden chamber.[15] Dilutions of **2** were added with the CCL2 (**2** + CCL2) in the lower chamber or with the cells in the upper chamber (**2**/CCL2). Following a 1.5 h incubation at 37 °C in a tissue culture incubator, the membrane was recovered from the microBoyden chamber, unmigrated cells on the upper surface were removed, and the migrated cells on the lower membrane surface were stained using a 3 step Dif-Quick stain.[15] The number of migrated cells was determined by microscopic evaluation using object recognition software (Bioquant, Nashville TN). Six 400× magnification fields were quantitated for each condition. Eighty percent of the normal donor population expresses the CCR2 variant, which requires 100 ng/mL CCL2 for maximum response; therefore monocyte migration toward 100 ng/mL CCL2 was used in this study[18] (and unpublished data, O.M.Z.H.). Specific binding was determined using the normalization protocols described in Graph Pad Prism (La Jolla, CA). In short, the mean background migration to media control (also labeled BM) was subtracted from all replicates, the maximum number of cells migrating to 100 ng/mL of CCL2 was set to 100%, and reductions in migration were calculated relative to the number of cells migrating to 100 ng/mL CCL2. The mean and standard error of the mean for each treatment ($N = 6$) are given.

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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