Small Neutralizing Molecules to Inhibit Actions of the Chemokine CXCL12

The chemokine CXCL12 and the receptor CXCR4 play pivotal roles in normal vascular and neuronal development, in inflammatory responses, and in infectious diseases and cancer. For instance, CXCL12 has been shown to mediate human immunodeficiency virus-induced neurotoxicity, proliferative retinopathy and chronic inflammation, whereas its receptor CXCR4 is involved in human immunodeficiency virus infection, cancer metastasis and in the rare disease known as the warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (WHIM) syndrome. As we screened chemical libraries to find inhibitors of the interaction between CXCL12 and the receptor CXCR4, we identified synthetic compounds from the family of chalcones that reduce binding of CXCL12 to CXCR4, inhibit calcium responses mediated by the receptor, and prevent CXCR4 internalization in response to CXCL12. We found that the chemical compounds display an original mechanism of action as they bind to the chemokine but not to CXCR4. The highest affinity molecule blocked chemotaxis of human peripheral blood lymphocytes ex vivo. It was also active in vivo in a mouse model of allergic eosinophilic airway inflammation in which we detected inhibition of the inflammatory infiltrate. The compound showed selectivity for CXCL12 and not for CCL5 and CXCL8 chemokines and blocked CXCL12 binding to its second receptor, CXCR7. By analogy to the effect of neutralizing antibodies, this molecule behaves as a small organic neutralizing compound that may prove to have valuable pharmacological and therapeutic potential.

Chemokines are small (8–10-kDa) secreted proteins that play roles in the normal physiology of the immune system as well as in orchestrating leukocyte recruitment and activation in the context of inflammatory and infectious diseases (1). Most of them belong to one of two major subfamilies: the β or CC chemokines in which two conserved cysteines from the amino terminus are adjacent to each other and the α or CXC chemokines in which these two cysteines are separated by one residue. Chemokine receptors are members of the superfamily of G protein-coupled receptors characterized by seven transmembrane-spanning regions and coupling to heterotrimeric G proteins.

The CXC chemokine stromal cell-derived factor-1 (SDF1), now named CXCL12, binds to and activates the chemokine receptor CXCR4 as well as the more recently identified CXCR7 receptor (19). CXCL12 stimulates a rapid receptor-mediated intracellular calcium mobilization and signaling through a Pertussis toxin-sensitive G protein. The response to CXCL12 and expression of the CXCR4 receptor occur at a very early stage of embryonic development and appear to be widely used whenever cell migration is required (2). Indeed mice lacking either CXCL12 or CXCR4 die prenatally and exhibit defects in vascular development, neuronal development, hematopoiesis, and cardiogenesis (3–6).

Besides the regulation of homeostatic processes, the CXCR4 receptor is implicated in tumor metastasis (7) as well as in infectious and inflammatory diseases. Indeed in different mouse models of allergic eosinophilic airway inflammation, it was shown that either competitive antagonists (8) or antibodies against the CXCR4 receptor as well as antibodies neutralizing the CXCL12 chemokine (9) significantly lower eosinophil recruitment in lung and reduce airway hyperreactivity.

Also inherited heterozygous autosomal dominant mutations of the CXCR4 gene, which result in the truncation of the carboxyl terminus (C-tail) of the receptor, are associated with the rare disease known as the warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis (WHIM) syndrome (10, 11).
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11). Finally the chemokine receptor CXCR4 also serves as a coreceptor to HIV type 1 to infect T cells (12).

Considering both qualitative and quantitative aspects of the involvement of the CXCR4/CXCL12 pair in the above mentioned physiological and pathological functions on the one hand and the limited number of pharmacological tools to investigate their function or to correct for defects in their functioning, we set up a screening program to identify new molecules interfering with the binding of CXCL12 to the receptor CXCR4. Here we describe the discovery of a new class of pharmacologically active molecules that bind to the chemokine itself and neutralize its biological activity in a way similar to that of neutralizing antibodies.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—All antibodies were purchased from BD Biosciences. Chalcone and baicalin stock solutions were prepared in sterile DMSO and then stored at –20 °C before use. The human chemokines CXCL12 and CXCL12-Texas Red were synthesized as described previously (13, 14). The strategy used for the introduction of the Texas Red molecule was the same as for the biotin molecule. After 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl probe, Texas Red was introduced using Texas Red dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl probe. The strategy used for the introduction of the Texas Red molecule was the same as for the biotin molecule. After 1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)-3-methylbutyl probe, Texas Red was introduced using Texas Red succinimidylerster, mixed isomers (Invitrogen). The human chemokines CCL5 and CXCL8 were purchased from BD Biosciences.

Chemical Library Screening—The collection of 3,200 screened molecules was taken from the Chemical Library of the School of Pharmacy of Strasbourg (Institut Fédératif de Recherche 85). Human embryonic kidney 293 cells expressing the fusion receptor EGFP-hCXCR4 (14) were harvested in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2PO4·7H2O, 1.4 mM KH2PO4, pH 7.4) supplemented with 5 mM EDTA, pH 7.4; centrifuged; and resuspended in Hepes-bovine serum albumin in a 1-ml quartz microcuvette. Fluorescence measurements were carried out as described previously (19) with the exception that CXCL12-biotin (CXCL12-biot) concentration was used here at 1 nM. The incubation of increasing concentrations of chalcone 4 with CXCL12-biot was made in the binding buffer during 1 h at room temperature before addition to cell suspension. Untagged CXCL12 was used at 1 μM as a control in the competition experiments.

Tryptophan Fluorescence Assay—Binding of chalcone 4 and chalcone 1 to CXCL12 was examined by monitoring changes in the emission intensity of intrinsic Trp fluorescence of the chemokine. Increasing amounts of molecule were added to CXCL12 protein (2 μM in Hepes buffer without bovine serum albumin in a 1-ml quartz microcuvette. Fluorescence measurements were carried out in triplicate using a Fluorolog 3 spectrofluorometer (Jobin-Yvon/SpeX). The excitation wavelength was set to 295 nm, and emission was collected from 310 to 400 nm. All fluorescence emission spectra were corrected for the Raman peak by subtracting the emission scan of the buffer alone.

Solubility Measurements—Solubility measurements of chalcones 1 and 4 were done by dissolving the compounds up to saturation in solutions of CXCL12 prepared in the following...
buffer: Tris 50 mM (pH = 8), NaCl 200 mM, CaCl2 1 mM, imidazole 10 mM. For each chalone, the maximal solubility was measured in four solutions containing 0, 312, 625, and 1000 μM CXCL12. Samples were shaken for 24 h at 20–22 °C, and for each solution, the saturation was confirmed by the presence of undissolved chalone in excess. After ultracentrifugation (Sorvall Discovery M120 SE ultracentrifuge with S45-A rotor centrifuged at 40,000 rpm), the concentration in the supernatant solution was determined using high performance liquid chromatography (HPLC).

The measurements were done using a Gilson HPLC chain with a UV detector set at 280 nm and a Rhodyne injector with a 50-μl loop. Data acquisition and processing were performed with Unipoint software version 1.71. The reverse phase preparations were carried out at room temperature on a 5-μm Luna C18(2) Phenomenex column (150 × 4.6 mm). The aqueous mobile phase contained 0.1% trifluoroacetic acid (solvent A). The organic phase was HPLC grade acetonitrile (Sigma-Aldrich CHROMASOLV) containing 0.1% trifluoroacetic acid (solvent B). The mobile phase flow rate was 1 ml/min, and the retention times for chalones 1 and 4 were 17.3 and 16.5 min, respectively. 50 μl were injected for the eight saturated solutions. The solutions with 625 and 1000 μM CXCL12 had to be diluted before HPLC analysis because their chalone concentrations were beyond the calibration ranges.

**Isothermal Titration Microcalorimetry**—Isothermal titration calorimetry measurements were carried out at 25.0 °C using a VP-ITC (MicroCal) titration calorimeter. All solutions were thoroughly degassed before use by stirring under vacuum. The sample cell was loaded with 1.4 ml of 1 μM CXCL12 in 50 mM Hepes, 100 mM KCl buffer, pH 7.5, and the reference cell contained distilled water. Titration was carried out using a 300-μl syringe filled with 0.2 mM chalone at 10% DMSO in Hepes/KCl buffer with stirring at 300 rpm. Injections were started after base-line stability had been achieved. A titration experiment consisted of 15 consecutive injections of 2-μl volume and 6.8-s duration for each with a 4-min interval between injections. The heat of dilution was measured by injecting chalone into buffer solution without protein. The enthalpy change for each injection was calculated by integrating the area under the peaks of recorded time course of power change and then subtracting that from the control titration. Data were analyzed using MicroCal Origin software with equations corresponding to sets of identical sites and to sets of independent sites.

**Mouse Model of Allergic Eosinophilic Airway Inflammation**—The protocol used BALB/c mice (9 weeks; Charles River, Saint-Germain-sur-l’Arbresle, France) according Ref. 20. Briefly mice were sensitized on days 1 and 7 by intraperitoneal injections of 50 μg of ovalbumin + 2 mg of Al(OH)3 in saline (phosphate-buffered saline) and challenged on days 18–21 by ovalbumin (10 μg intranasally, 12.5 μl/nasal). Chalone 4 (350 μmol/kg intraperitoneally) or vehicle (1% carboxymethylcellulose) was administered 2 h before each ovalbumin challenge. On day 22, the lungs were lavaged (10 × 0.5 ml of saline-EDTA). The bronchoalveolar lavage fluid was centrifuged to pellet cells, and erythrocytes were lysed by hypotonic shock. Cells were resuspended in 500 μl of ice-cold saline-EDTA. Total and differential cell counts were determined after cytocentrifugation of 50,000 cells/slide and Hemacolor (Merck) staining. At least 400 cells were counted and identified as macrophages, eosinophils, lymphocytes, or neutrophils as expressed as an absolute number from the total cell count.

**Modeling of SDF1-Chalone 4 Complex Three-dimensional Structure**—The 5.26 release of the Cambridge Structural Database (21) was searched to retrieve the crystal structures of chemically similar compounds. The naked chalone scaffold of chalone 4 was used as Conquest query. The 2006 release of the screening Protein Data Bank (22) was searched to retrieve the crystal structure of chalone 4 chemical analogs bond to protein. The three-dimensional structure of 2’,4’,4”-trihydroxychalcone in complex with the chalone o-methyltransferase (Protein Data Bank code 1FP1) was edited in Sybyl (Tripos, Inc., St. Louis, MO) to generate chalone 4 coordinates stored in the MOL2 file. Hydrogens were added according to the Ichem (ChemAxon Kft., Budapest, Hungary) preferred tautomer at physiological pH.

A few rotameric states were modified in the monomeric structure of CXCL12 (Protein Data Bank code 1VMC) to enlarge the existing cleft at the dimer interface. The largest changes concerned Leu-26 (χ1 moved from gauche− to gauche+, and χ2 moved from trans to gauche−), Ile-58 (χ1 moved from gauche− to gauche+), Tyr-61 (χ2 moved from gauche+ to gauche−), and Leu-62 (χ1 moved from gauche− to trans, and χ2 moved from trans to gauche−).

Docking experiments were carried out using Gold (Cambridge Crystallographic Data Centre, Cambridge, UK). Generic algorithm default parameters were set, and the Goldscore scoring function was chosen. The protein site was defined with a radius of 10 Å around a point in the center of the cavity. Two distance restraints of 1.5-4.5 Å with a spring constant of 5 were set between the halogen atom of the chalone 4 chlorophenyl moiety and Ile-51 and Trp-57 side chains. The chalone 4 best pose was manually edited to solvent expose the ligand carbonyl group (which was buried in the hydrophobic region of the protein) and to fix unrealistic torsion angles around the vinyl group. The optimized complex between SDF1 and chalone 4 was further energy-minimized using Sybyl (default settings) and served as the target for chalone 4 docking.

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**RESULTS**

Searching for Small Compounds That Could Inhibit the Interaction of the Chemokine CXCL12 with Its CXCR4 Receptor—We screened 3,200 molecules from the collection of the medicinal chemistry laboratories from Strasbourg University in a fluorescent binding assay on whole living cells described previ-
was reached at concentrations beyond 300 nM, and the dissociation of Texas Red-labeled CXCL12 (100 nM) to EGFP-tagged CXCR4. Normalized EGFP emission intensity at 510 nm (24). Screening of this collection to find inhibitors of the interaction that we observed points to the importance of substitution of ring A by the chloride atom at position 4′ and to the simultaneous substitutions at positions 3 and 4 of ring B (data not shown). The affinity of chalcone 4 is only 1 order of magnitude lower than that of the reference competitive antagonist peptide T134 (25) (Fig. 1B and Table 1). The unsubstituted chalcone 1 is also present in the collection. However, because of its very weak affinity, the molecule was not identified as a hit compound.

**Chalcone 4 Inhibits CXCL12-evoked Calcium Cellular Responses**—The next step toward pharmacological characterization of the most potent compound, chalcone 4, consisted in determining its effects on CXCR4-mediated cellular responses. Chalcone 4 by itself did not induce any calcium response (data not shown).

Fig. 1C shows that chalcone 4 inhibited CXCL12-evoked calcium responses in a dose-dependent manner and with an apparent inhibitory constant (210 ± 50 nM) that is in good agreement with its potency for inhibition of CXCL12 binding to its receptor by chalcone 4. The structure-activity relationship that we observed points to the importance of substitution of ring A by the chloride atom at position 4′ and to the simultaneous substitutions at positions 3 and 4 of ring B (data not shown). The affinity of chalcone 4 is only 1 order of magnitude lower than that of the reference competitive antagonist peptide T134 (25) (Fig. 1B and Table 1). The unsubstituted chalcone 1 is also present in the collection. However, because of its very weak affinity, the molecule was not identified as a hit compound.

**Chalcone 4** is an analog of the low affinity chemical platform chalcone 1 that is devoid of side chains (IC₅₀ > 500 μM). As the two aromatic rings progressively become more substituted (chalcone 2, chalcone 3, and chalcone 4), the dissociation constants incrementally decreased to reach a submicromolar value (IC₅₀ = 150 ± 50 nM for chalcone 4; see Fig. 1A for an example of the inhibition of the association of chalcones, namely chalcone 2, chalcone 3, and chalcone 4, of chalcones, three molecules, that we observed points to the importance of substitution of ring A by the chloride atom at position 4′ and to the simultaneous substitutions at positions 3 and 4 of ring B (data not shown). The affinity of chalcone 4 is only 1 order of magnitude lower than that of the reference competitive antagonist peptide T134 (25) (Fig. 1B and Table 1). The unsubstituted chalcone 1 is also present in the collection. However, because of its very weak affinity, the molecule was not identified as a hit compound.

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Yet in contrast to the known competitive peptide T134 that fully blocks calcium signaling at high concentration (20 µM; data not shown), chalcone 4 did not block more than 60–70% of the response to 5 nM CXCL12. Maximal inhibition by chalcone 4 was not improved when preincubation duration with cells was increased from typically 30 s to 30 min, supporting our view that the mechanism of inhibition by chalcone 4 differs from that of peptide T134.

Chalcone 4 Inhibits CXCL12-evoked CXCR4 Internalization—As an antagonist of CXCR4 responses, chalcone 4 also altered chemokine-induced receptor internalization (Fig. 1B). Receptor endocytosis was monitored on HEK cells expressing EGFP-CXCR4 and quantified by flow cytometry. Endocytosis was time-dependent and reached 55 ± 4% in 30 min when cells were exposed to 200 nM CXCL12. Chalcone 4 (1 µM) did not alter the level of surface receptor on its own but significantly reduced the CXCL12 effect because only 20 ± 8% of the receptor molecules were internalized in 30 min.

Chalcone 4 Selectivity among Chemokine Receptors—To gain insight into compound selectivity, we next characterized the effect of chalcone 4 on calcium responses of various chemokines/receptor pairs (Fig. 2). Consistent with data from Fig. 1C, chalcone 4 inhibited 50% of CXCL12-evoked calcium responses in HEK EGFP-CXCR4 cells (Fig. 2, left panel). In contrast, it had no effect on CCL5-evoked calcium responses in HEK CCR5 cells (Fig. 2, middle panel) and inhibited only 15% of the maximal CXCL8-evoked responses in HEK EGFP-CXCR1 cells (Fig. 2, right panel). These results support the idea that chalcone 4 shows selectivity for the CXCL12/CXCR4 pair.

Chalcone 4 Inhibits Chemotactic Responses to CXCL12—in contrast to other known antagonists of CXCR4 receptors, such as T134, P2G-CXCL12, or AMD3100 (26–28), we found that chalcone 4 does not inhibit infection of human CD4+ CXCR4+ T lymphocytes by HIV in an assay carried out as described previously (29) (data not shown). In such an assay, there is no implication of the chemokine CXCL12. The data could be

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**TABLE 1**

| Compound                        | Structure | IC50     |
|---------------------------------|-----------|----------|
| Chalcone 1 or (E)-1,3-diphenyl(prop-2-en-1-one) | ![Structure](image1.png) | IC50 > 500 µM |
| Chalcone 2 or (E)-3-(4-hydroxyphenyl)-1-phenyl(prop-2-en-1-one) | ![Structure](image2.png) | IC50 = 15 ± 3 µM |
| Chalcone 3 or (E)-1-(6'-hydroxyphenyl)-3-(4-hydroxyphenyl)prop-2-en-1-one | ![Structure](image3.png) | IC50 = 24.2 ± 5 µM |
| Chalcone 4 or ((E)-1-(4'-chlorophenyl)-3-(4-hydroxy-3-metoxyphenyl)prop-2-en-1-one) | ![Structure](image4.png) | IC50 = 150 ± 50 nM |
| T134 RRWCYRKDPYRCiCR-COOH | ![Structure](image5.png) | IC50 = 6.9 ± 0.5 nM |
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FIGURE 2. Selectivity of chalcone effects. Shown is inhibition by 10 µM chalcone 4 of calcium responses triggered by either 10 nM CXCL12 on HEK EGFP-CXCR4-expressing cells, or by 50 nM CXCL8 on HEK EGFP-CXCR1-expressing cells. Typical kinetics of the calcium response recorded during 80 s are shown. Results are expressed as the percentage of the maximal peak amplitude of the calcium response in the absence (black bars) or in the presence of 10 µM chalcone 4 (gray bars) and represent the mean ± S.D. of three independent experiments. Statistical analysis consisted of unpaired two-tailed Student’s t tests and was conducted with Prism software (GraphPad), *, p < 0.05; **, p < 0.005.

interpreted if one proposes that chalcone 4 binds to the chemokine CXCL12 instead of directly binding to the CXCR4 receptor. There is precedent because the natural flavone isolated from Scutellaria baicalensis, baicalin, has been shown to bind, although with low affinity, to various chemokines (30), and interestingly, chalcones are precursors of flavones and anthocyanins. We did confirm that baicalin is an inhibitor of CXCL12 binding to CXCR4 (Fig. 1B) although weaker than chalcone 4. We then decided to further explore the mode of action of chalcone 4 using complementary experimental approaches.

We first investigated the effect of chalcone 4 on the chemotactic activity of CXCL12. The assay, carried out with CD4+ enriched T cells isolated from human blood, revealed a drastic difference depending on the protocol. Indeed when chalcone 4 (0.5, 2, or 10 µM) was preincubated with the cells, no inhibition of CD4+ T cell chemotaxis was detected (Fig. 3A), whereas the specific CXCR4 antagonist, AMD3100 (1 µM), inhibited 90% of CD4+ T cell chemotaxis (18). In contrast, when chalcone 4 was preincubated with CXCL12 (Fig. 3A), we observed a dose-dependent inhibition of chemotaxis with an apparent affinity close to 1 µM suggesting that chalcone 4 may bind to the chemokine rather than to the receptor. The absence of effects of chalcone 4 alone on cells showed in addition that the molecule is non-toxic to human T lymphocytes and does not trigger chemotaxis on its own.

Chalcone 4 Inhibits CXCL12 Binding to CXCR7—If chalcone 4 acts as a ligand of the CXCL12 chemokine, one straightforward prediction related to this mechanism of action is that binding of CXCL12 to its second natural receptor, CXCR7 (19, 31), should be blocked by chalcone 4 as well. We tested this hypothesis in a binding assay making use of fluorescence detection of bound CXCL12-biot by flow cytometry (19). A0.01 cells that do not express CXCL12 binding sites were transfected with CXCR7. They displayed significant CXCL12-biot binding (Fig. 3B) of which nearly 80% was displaced by unlabeled CXCL12 in excess. If chalcone was used to prevent CXCL12-biot binding, a clear dose-dependent inhibition was detected (Fig. 3B). The efficacy of chalcone 4, however, was strongly enhanced if the chemokine was preincubated with the chalcone for 1 h. At the concentration that blocks 50% of the binding of CXCL12 to CXCR4, namely 200 nM, chalcone 4 also displaced 50% of specific CXCL12 binding to CXCR7. This effect was clearly detected if the chalcone was preincubated with the chemokine.
as was shown to be necessary in the chemotaxis assay. Chalcone 4 thus inhibited binding of CXCL12 to both CXCR4 and CXCR7 receptors with similar affinity, supporting the notion that the molecule interacts with the chemokine and neutralizes its binding capacity.

**Chalcone 4 Binds to the Chemokine CXCL12 but Not to CXCR4**—The second evidence supporting that chalcone 4 binds to CXCL12 was provided by tryptophan fluorescence analysis. CXCL12 contains a single tryptophan residue, Trp-57. This amino acid belongs to the carboxyl-terminal helical domain of the chemokine, and its indole ring is buried in a hydrophobic pocket (32). When CXCL12 is incubated with increasing concentrations of chalcone 4, we found that tryptophan fluorescence intensity at 340 nm declined (Fig. 4A). The resulting Trp fluorescence inhibition curve was satisfactorily fitted according to a 1:1 stoichiometry interaction model. The deduced dissociation constant, \( K_D = 220 \pm 80 \text{ nM} \), was in the same range as the affinity estimates derived from CXCL12-Texas Red binding assays. Also consistent with the binding assays shown in Fig. 1B, the analog chalcone 1 devoid of substituent groups displayed poor potency to alter Trp fluorescence and weak affinity (\( K_D > 50 \text{ \mu M} \)).

**Chalcone 4 Binds to Multiple Sites on CXCL12**—While determining physicochemical properties of chalcones, we noticed that they exhibit poor solubility in physiological buffers (5–15 \( \mu M \) maximal solubility) and that solubility was significantly improved by soluble proteins like serum albumin. In addition, we noticed that the actions of the chalcones were significantly larger after preincubation of the molecule with the target protein. We therefore addressed the question as to whether the chemokine CXCL12 was able to solubilize the chalcone molecules. Solubility of chalcones 1 and 4 was thus determined after 24-h incubation in a physiological buffer containing various amounts of CXCL12. Fig. 4B shows that CXCL12 was extremely efficient in solubilizing chalcone molecules because experimental values are in the millimolar range in the presence of chemokine. Interestingly the stoichiometry of solubilization approaches three molecules of chalcone 4 solubilized by one molecule of CXCL12, whereas only about two molecules of chalcone 1 are solubilized per CXCL12 molecule. This experiment strongly argues in favor of chalcone molecules interacting with CXCL12 with, in addition, chalcone 4 binding to one supplementary site presumably mediating the biological effect and accounting for tryptophan fluorescence quenching.

The fact that chalcone molecule solubilization results from binding to CXCL12 chemokine was further confirmed by isothermal titration microcalorimetric measurements. Fig. 5 shows a heat effect generated by addition of chalcones 1 and 4 to solutions of CXCL12, reflecting differential behaviors of the two molecules. The shapes of the two titration curves are similar for molar ratios beyond 2, i.e. for modest to low affinity interaction, and exhibit an additional component in the molar ratio smaller than 2 for chalcone 4 only, indicating some high
affinity interaction. Data were fitted using the equations for one or two categories of independent site(s). The best fits were obtained for each chalcone molecule with the number of sites estimated from the solubility experiment stoichiometries, indicating that chalcone 1 (Fig. 5B) binds to two identical sites with low affinity ($K_D = 5.5 \pm 0.3 \times 10^{-6}$ M), whereas chalcone 4 (Fig. 5A) binds to one high affinity site ($K_D = 1.7 \pm 0.3 \times 10^{-8}$ M) and two low affinity sites ($K_D = 2.1 \pm 0.2 \times 10^{-6}$ M).

**Chalcone 4 Reduces Inflammation in a Murine Model of Allergic Eosinophilic Airway Inflammation**—Altogether the results show that chalcone 4 directly interacted with CXCL12, reduced the ability of this chemokine to bind to its CXCR4 receptor, and thus partially neutralized the biological functions of the chemokine/receptor pair in vitro. To extend this finding in an in vivo model involving CXCR4, we analyzed the effects of chalcone 4 in a model of allergic eosinophilic airway inflammation (8, 9).

CXCL12 and CXCR4 have been suspected to take part in inflammatory processes in particular because CXCR4 is associated with neutrophils, monocytes, and mast cells, which are involved in asthma-associated immune responses. We therefore investigated the potential of chalcone 4 to inhibit airway inflammation in a murine model of ovalbumin-induced allergic eosinophilic airway inflammation. Mice were sensitized to and challenged with ovalbumin-sensitized and -challenged mice treated either with 350 $\mu$g/kg chalcone 4 or with its vehicle alone (carboxymethylcellulose). Data are means ± S.E. of $n = 6–7$ animals. *, $p < 0.05$.

**DISCUSSION**

In this work we provide convergent functional and biophysical data to show that a low molecular weight molecule is able to bind to the chemokine CXCL12 with high affinity to prevent binding of the chemokine to the receptors CXCR4 and CXCR7 and thus to alter the functional consequences of this interaction as demonstrated here by inhibition of ex vivo chemotaxis and in vivo anti-inflammatory activity in the airways. Considering that chemokines are rather small proteins, our observations raise questions concerning the molecular mechanism of action of the neutralizing molecule chalcone 4 and about the location of a suitable site(s) for small chemicals. Three crystallographic and three NMR structures of CXCL12 are available in the Protein Data Bank (1QG7 (34), 1A15 (32), 2SDF and 1SDF (35), 2SDF (35), and 1VMC (36)). In all structures, CXCL12 adopts a typical fold comprising an $\alpha$-helix tightly packed with a three-strand antiparallel $\beta$-sheet.

In the monomeric state, the CXCL12 fold has been characterized by NMR. It is almost identical to that reported in the dimeric state. In the most acute monomer NMR structure available (Protein Data Bank code 1VMC), the CXCL12 hydrophobic patch consists of a shallow depression at the surface of the protein. Minor conformation changes allow us to form a pocket that perfectly accommodates chalcone 4 (the overall root mean square deviation computed over all residue $\alpha$-atoms of CXCL12 of the starting and optimized structure is only 0.24 Å). The entire pocket, up to its mouth, is hydrophobic.

In the modeled CXCL12-chalcone complex, the chlorophenyl moiety of the chalcone, which appears to be critical for high affinity binding, is deeply buried inside the pocket (Fig. 7), and the $3'$-methoxy, $4'$-hydroxyphenyl moiety slightly protrudes from the solvent-accessible face of the cavity, thereby plausibly occluding the protein dimerization site.
We thus considered the possibility that chalcone 4 interacts with the monomer of CXCL12. Veldkamp et al. (37) and others (38) investigated the effect of solution composition on the quaternary structure of CXCL12, and they showed that CXCL12 exists in a monomer-dimer equilibrium, yet only under extreme conditions, and that the dimer dissociation $K_D$ is highly dependent on both the solution pH and the presence of stabilizing counterions. Specifically for CXCL12 dimerization to occur, high chemokine concentrations are required (i.e., the $\mu$M–mM range); multivalent anions like phosphate, sulfate, citrate, or heparin must be present; and the pH must be above the presumed $pK_a$ of His-25, a residue positioned at the interface of the dimer (32, 34, 35). In samples containing only Heps buffer at pH 7.4 the dimer dissociation $K_D$ is beyond 10 mM, whereas in 100 mM sodium phosphate at pH 7.4, it is 140–180 mM (37, 38). Accordingly in our experimental conditions binding and functional responses were measured at 2 orders of magnitude lower concentrations than those at which CXCL12 can dimerize. The chemokine CXCL12 is thus most likely in a monomeric state in our study.

The very short distance (around 4 Å) between the Trp indole and chalcone 4 in the model is also consistent with the ligand being able to quench Trp fluorescence. Accordingly the modeled site would correspond to the high affinity site at the level of which chalcone 4 blocks chemokine function. On the other hand, it has been described that small molecules may be accommodated into unexpected pockets, arising from adaptative processes, that could not be predicted on the basis of crystallographic data from protein (39). Further structural studies, including co-crystallization of the NMR solution structure of the complex, will thus be required to refine structural hypotheses.

Chalcone 4 exhibited binding selectivity for the chemokine CXCL12 as compared with CCL5 and CXCL8. Although the overall fold is conserved among the three proteins (supplemental Fig. 1A), changes are observed in the carboxyl-terminal helix orientation with respect to the β-sheet especially between CCL5 and CXCL12. Residues forming the hydrophobic surface patch of CXCL12 and CXCL8 are very similar, but the putative binding pocket in CXCL8 is larger than the one in CXCL12. Thus chalcone 4 could interact in a different manner with these two chemokines. CCL5 has a higher content of aromatic amino acid residues with a higher compactness of side chains. As a consequence, CCL5 hydrophobic incurvation is shallower than that in CXCL8 and CXCL12 (supplemental Fig. 1B), and it is unlikely to bind any small molecular weight compound. The hydrophobic incurvature on CXCL8 is larger than the hydrophobic incurvature on CXCL12, and the chalcone could interact in a different manner.

Chalcones constitute a relatively large group endowed with potential therapeutic biological activities on analgesia, inflammation (40–43), infectious diseases (44–46), or cancer (47). In the majority of plants, chalcones are precursors of other classes of flavonoids, such as flavanones, dihydroflavonols, and finally anthocyanins, the major water-soluble pigments in flowers and fruits (for a review, see Ref. 48). The flavone baicalin has received attention because of its potential anti-inflammatory actions and inhibitory effect on HIV (49–51) reverse transcriptase, a mechanism by which baicalin is thought to be active at inhibiting HIV infection (49). The action of baicalin as an anti-inflammatory agent may proceed through its interaction with chemokines as suggested by Li et al. (30). However, it must be noticed that the affinity of baicalin for the chemokines CCL8, CXCL8, CCL4, or CXCL12 is very weak (30), and we did confirm it in this work with CXCL12.

Considering that chalcone 4 exhibits potent anti-inflammatory activity upon binding to CXCL12 and not to CXCR4, its mechanism of action markedly differs from that of other pharmacological agents acting upon binding to the receptor CXCR4, such as AMD3100 (52), ALX40-4C, or T22/T140 (5, 53, 54). Analysis of functional properties of these compounds on constitutively active mutants of CXCR4 receptors (55) reveals that, at high doses, AMD3100 and ALX40-4C are weak partial agonists and that T22/T140 is an inverse agonist of the receptor functions.
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As a consequence of partial agonism, both AMD3100 and ALX40-4C molecules are cautiously considered as antimetastatic agents in the many cancer types involving CXCR4 (56, 57). Thus, it is possible that none of the known CXCR4 antagonists will be found to have a neutral effect on basal CXCR4 response levels.

Chalcone 4, in contrast to AMD3100, ALX40-4C, and T22/T140, did not alter the resting/basal level of the CXCR4-associated responses, as we show here on both calcium and chemotactic responses. It behaved as a neutral inhibitor of the ligand.

T140, did not alter the resting/basal level of the CXCR4-associated response levels.

Thus, it is possible that none of the known CXCR4 antagonists will be found to have a neutral effect on basal CXCR4 static agents in the many cancer types involving CXCR4 (56, 57).

Chemokine Neutraligands

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