Selective Binding and Oligomerization of the Murine Granulocyte Colony-stimulating Factor Receptor by a Low Molecular Weight, Nonpeptidyl Ligand*

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Granulocyte colony-stimulating factor regulates neutrophil production by binding to a specific receptor, the granulocyte colony-stimulating factor receptor, expressed on cells of the granulocytic lineage. Recombinant forms of granulocyte colony-stimulating factor are used clinically to treat neutropenias. As part of an effort to develop granulocyte colony-stimulating factor mimics with the potential for oral bioavailability, we previously identified a nonpeptidyl small molecule (SB-247464) that selectively activates murine granulocyte colony-stimulating factor signal transduction pathways and promotes neutrophil formation in vivo. To elucidate the mechanism of action of SB-247464, a series of cell-based and biochemical assays were performed. The activity of SB-247464 is strictly dependent on the presence of zinc ions. Titration microcalorimetry experiments using a soluble murine granulocyte colony-stimulating factor receptor construct show that SB-247464 binds to the extracellular domain of the receptor in a zinc ion-dependent manner. Ultrastructural studies demonstrate that SB-247464 induces self-association of the N-terminal three-domain fragment in a manner that is consistent with dimerization. SB-247464 induces internalization of granulocyte colony-stimulating factor receptor on intact cells, consistent with a mechanism involving receptor oligomerization. These data show that small nonpeptidyl compounds are capable of selectively binding and inducing productive oligomerization of cytokine receptors.

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Granulocyte colony-stimulating factor (G-CSF)1 is the primary cytokine controlling proliferation and differentiation of myeloid cells along the granulocytic pathway (1). Lack of G-CSF results in a profound neutropenia and increased susceptibility to infections (2). Recombinant G-CSF is widely used to stimulate neutrophil production in patients suffering from neutropenia as a result of chemotherapy or bone marrow transplantation and is also used to mobilize hematopoietic precursor cells into the periphery prior to leukopheresis (3, 4).

G-CSF is a member of the four-helix bundle family of cytokines and binds to a type I cytokine receptor expressed on the surface of neutrophils, granulocytic cells, and granulocytic precursors (5). Binding of G-CSF to its receptor promotes oligomerization of receptor chains, triggering the activation of JAK kinases associated with the cytoplasmic region of the receptor (6–9). Activated JAKs then phosphorylate specific tyrosine residues on a number of signaling proteins including STAT3, STAT5, and SHC, which ultimately results in changes in cell phenotype (7–9).

Type I cytokine receptors are defined by the presence of a CRH region, which contains four conserved cysteine residues and a WSXWS motif (8, 10, 11). The extracellular domain of some receptors, such as those for erythropoietin and growth hormone, consist solely of a CRH domain, which comprises the ligand binding site. Crystal structures showing cytokines bound to CRH domains indicate that the CRH region consists of two FN-III-like domains angled at ∼90° with respect to each other (12–14). Both structural and mutagenesis data show that the cytokine interacts primarily with amino acids in loop regions at the ends of the FN-III-like domains that project into the space formed at the domain junction (14).

The extracellular domain of the G-CSF receptor also contains a CRH region, which is separated from the membrane by three FN-III-like repeats (15, 16). These repeats are not involved in ligand binding but may play a role in stabilizing receptor dimers (16). A further Ig-like domain is attached to the N terminus of the CRH region, completing the six-domain structure of the G-CSF receptor extracellular region. Biophysical characterization of soluble proteins containing CRH domains alone or CRH- and Ig-like domains show that both CRH and Ig-like domains play a role in binding G-CSF (17, 18). G-CSF causes dimerization or higher order oligomerization of the receptor subunits and of the intact G-CSF receptor extracellular domain (19). G-CSF receptor activation is dependent on the presence of zinc ions that are believed to mediate receptor dimerization (20, 21).
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EXPERIMENTAL PROCEDURES

Luciferase Assay—Cytokine-independent NFS60 cells containing a stably integrated STAT-responsive luciferase reporter gene that is induced by G-CSF have been described previously (23). The cells were grown in RPMI containing 10% FBS and split to a density of 2 × 10^6/ml 2 days before the assay. Cells were transferred to RPMI containing 0.5% FBS and 100-μl aliquots containing 1 × 10^5 cells incubated with G-CSF (10 ng/ml) or SB-247464 in the presence of EDTA and metal chloride as indicated. After 2.5 h, SB-247464 was solvated in MeSO. The final MeSO concentration in the assay was maintained at 0.3%. At the end of the incubation period, the medium was removed from the cells, which were then lysed, and luciferase levels were determined using a luminometer. In some cases, results are graphed as relative luciferase units.

Production of Soluble Murine G-CSF Receptor Proteins—The extracellular domain of the murine G-CSF receptor comprising amino acids 1–626 (15) was expressed in CHOElA cells as an Fc fusion protein (G-CSFR-Fc). The N- and C-terminal three-domain fragments of the receptor, IgCk1Ck2 (amino acids 1–332) and FcNFnFn (amino acids 334–626), respectively, were also expressed in CHOElA as Fc fusion proteins but contained an FXa cleavage site to allow generation of monomers by proteolytic removal of the Fc fragment (see Fig. 3). Fc proteins were purified by protein G affinity chromatography followed by size exclusion chromatography on a Superdex 200 column. Monomer fragments were made from the dimeric Fc fusion proteins by incubation with FXa, a second protein G column, and a sizing step with Superose 200. Purity of the products was evaluated by Coomassie Blue staining of samples subjected to SDS-PAGE and was found to be better than 85% for the G-CSFR-Fc protein and better than 90% for the monomeric IgCk1Ck2 and FcNFnFn proteins. N-terminus sequences were confirmed for all the proteins.

Circular Dichroism Studies—Circular dichroism measurements were made with a Jasco J-710 1 CD spectropolarimeter at 0.3 mg/ml protein concentration in a 0.1-cm water-jacketed cuvette. Wavelength scans were made at 50 nm/min, and several spectra were recorded and averaged.

Isothermal Titration Calorimetry (ITC)—Measurements were made with a VP-ITC instrument (MicroCal, Northampton, MA) (24, 25). Dissociation constants and ligand concentrations were degassed for 10 min prior to each titration. Receptor concentrations were determined by absorbance at 280 nm using extinction coefficients calculated from the amino acid sequences (26). Ligand concentrations were determined gravimetrically. Titrations were carried out with injection volumes of 5–10 μl and a time interval between injections of 200 s. A preliminary injection of 2 μl was made before each titration to ensure the titrant concentration was at its loading value. Binding isotherms were fitted by nonlinear regression using an assumed 1:1 stoichiometric model provided in the Origin ITC software (MicroCal) (24). Although all of the reactions characterized involved multiple equilibria (e.g. multiple SB-247464:ligand stoichiometric species, overall binding reactions between SB-247464, an Fc receptor to form ternary complex), there was not enough information in the data to permit analysis by more complex models. Nevertheless, the simple model used was found to describe all of the data to within experimental uncertainty, and the resulting fitting parameters (reported as empirical “observed” parameters) provide a useful quantitative measure of overall binding affinity and thermodynamics for comparative purposes. The empirical fitting parameters, reported as observed parameters, are as follows: the binding molar ratio (N), dissociation constant (K_1), and enthalpy change (ΔH_m). Due to the very low solubility of SB-247464, experiments were conducted in buffer containing 3% MeSO plus 30% sucrose, which was found empirically to enable SB-247464 to be dissolved up to 150 μM.

Analytical Ultracentrifugation—Sedimentation equilibrium experiments were measured on a Beckman XL-A analytical ultracentrifuge. Samples were loaded in double sector cells with charcoal-filtered eppendorf centerpieces and sapphire windows. Samples were centrifuged until equilibrium was attained, as judged by an unchanging absorbance versus radial position profile. Sedimentation data were analyzed with a single species model as in Ref. 27 with either a single, homogeneous species model (Equation 1) or a monomer-dimer model (Equation 2).

A_{diss} = c_0 + c_m \exp[-r \cdot (M + H)/0] \exp[2d] + offset (Eq. 1)

A_{diss} = c_0 + c_m \exp[-r \cdot (M + H)/0] \exp[2d] + offset (Eq. 2)

Here σ = (M − r)^2/r^2 \exp[2d] and K_m is the dimerization equilibrium dissociation constant. A_{diss} represents the absorbance at the detection wavelength λ and radial position r, c_m is the concentration of...
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SB-247464

SB-250017

Fig. 1. Structure of metal complexes of SB-247464 and SB-250017. SB-247464 is arbitrarily shown with the exocyclic imine bonds in the trans configuration. Coordination of metal ions by the chelating nitrogens in the compounds is indicated by the dashed lines. Solid lines indicate potential additional coordination of the metal ion to other ligands (L). Note that a dimeric complex of SB-247464 hold together by two metal ions can also be formed.

monomer at the meniscus, and \(\varepsilon_l\) is the molar extinction coefficient/mole of monomer at wavelength \(\lambda\). \(M\) and \(v\) are the molecular weight and partial specific volume of a monomer subunit, \(\rho\) is the solvent density, \(\omega\) is the angular velocity, \(r_p\) is the radial position at the meniscus (in centimeters), \(R\) is the universal gas constant, \(T\) is the absolute temperature, and \(v_{offset}\) is a constant offset fitting parameter.

In the conditions of the present study, the solution density was \(\rho = 1.1173\ g/ml\) and \(v_{offset}\) values were calculated from sequence as 0.7364 and 0.7387 ml/g, respectively, for IgCk1Ck2 and FnFnFn monomer constructs. The floating parameters in Equation 1 are \(M,\ c_{offset}\) and \(v_{offset}\).

Detection of Activated STATs by Electrophoretic Mobility Shift Assay—NFS60 cells were grown and treated with G-CSF, SB-247464, and/or SB-250017 using the conditions described above, except that 5–10\(^6\) cells were used per treatment, and the incubation period was 30 min. At the end of the incubation, the cells were lysed in 25 \(\mu\)l of lysis buffer (20 mM Heps, pH 7.9, 300 mM NaCl, 10 mM KCl, 1 mM MgCl\(_2\), 0.1% Triton X-100, 0.5 mM dithiothreitol, 0.2 mM phenabclo, 20% glycerol, protease inhibitors, 2 mM NaVO\(_4\)) to give a whole cell extract. Electrophoretic mobility shift assay reactions using 3 \(\mu\)l of this extract and a labeled RIF-1 oligonucleotide STAT binding site were performed as described (28).

Internalization Assays—NFS60 cells were grown as described above. 1 \(\times\) 10\(^6\) cells were incubated with G-CSF or with SB-247464 for 2 h at 37 °C in RPMI containing 0.5% FBS. Cells were then washed three times in ice-cold PBS and resuspended in PBS. Cells were stained on ice by incubation with a 0.45 \(\mu\)g/ml concentration of a G-CSF-phycoerythrin fusion protein (R & D Systems, Minneapolis, MN) for 20 min and then washed three times in ice-cold PBS and resuspended in PBS. Cell staining was quantitated using a fluorescence-activated cell sorter (BD Biosciences). To test the effect of SB-247464 on G-CSF-phycoerythrin fusion protein (R & D Systems, Minneapolis, MN) for 20 min.

Transient Transfection Assays—The human and mouse G-CSF receptor expression constructs have been described previously (23). The chimeric receptors encode the following amino acids: M/H1, mouse amino acids 1–612, human amino acids 612–707; M/H2, mouse amino acids 1–601, human amino acids 601–707; M/H3, mouse amino acids 1–584, human amino acids 582–707. HepG2 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS. Cells were co-transfected with the indicated G-CSF receptor expression construct and a luciferase reporter under the control of a multimterized STAT-binding element, 4×1RIFtklc (23). Cells were transfected in triplicate in 24-well plates using Superfect (Qiagen Inc., Chatsworth, CA), as instructed by the manufacturer. 48 h after transfection, cells were treated with 10 ng/ml G-CSF or 1 \(\mu\)M SB-247464 for 4 h prior to lysis and determination of luciferase activity as described above.

RESULTS

SB-247464 Activity Requires Zinc Ions—Inspection of the structure of SB-247464 showed a rigid 5,5 bicyclic system resulting from the linkage of two opposing 2-guanidinobenzimidazole fragments. These functional groups, together with the presence of the two pyridines at the ring junctions, suggest a potential for the molecule to interact with metal ions through coordination with the sp\(^2\) lone pairs of three adjacent nitrogen atoms, as shown in Fig. 1. They also allow for the formation of a dimer of SB-247464 linked by two metal ions. Both this type of tridentate coordination and dimer formation have actually been demonstrated by x-ray crystallographic studies of a number of metal complexes derived from SB-247464.2 To test for an effect of metal ion concentration on compound activity, we incubated SB-247464 in tissue culture medium containing EDTA, a metal chelator, at various concentrations, and assayed for compound activity using a G-CSF-inducible reporter cell line. Incubation of this cell line with G-CSF or with SB-247464 alone causes a readily detectable increase in luciferase expression (Fig. 2A); for SB-247464, maximal expression of luciferase took place at a concentration of 1 \(\mu\M. Activation of luciferase by 1 \(\mu\M SB-247464 was progressively decreased in the presence of increasing concentrations of EDTA (Fig. 2B). In this concentration range (0.3–100 \(\mu\M\)), EDTA had no effect on luciferase induction in response to G-CSF, indicating that the elimination of the SB-247464 response is not due to a nonspecific effect on luciferase expression or cell viability (Fig. 2C). The activity of 1 \(\mu\M SB-247464 in the presence of 50 \(\mu\M EDTA could be recovered by titrating in additional zinc (II) ions (Fig. 2D). The reduction in luciferase activity at higher concentrations of Zn(II) is due to a nonspecific toxicity of Zn(II) at these concentrations. Titrating in other metal ions such as Mn(II) and Fe(II) (Fig. 2D) or Cu(II), Ni(II), or Co(II) (data not shown) did not restore the original activity elicited by SB-247464, indicating a selective requirement of zinc ions for the G-CSF activity.

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Conditions were as follows: 10 mM HEPES, pH 7.3, 150 mM NaCl, 30% sucrose, 0.1 cm path length, and 0.3 mg/ml receptor protein. Molecular masses of the purified IgCk1Ck2 and FnFnFn-Fc fragments (Fig. 3C) were determined by gel filtration chromatography and ionization mass spectrometry to be 238,258 Da (G-CSFR-Fc), 44,939 Da (IgCk1Ck2), and 47,087 Da (FnFnFn). Secondary structures of the IgCk1Ck2-Fc and FnFnFn-Fc constructs were found to be indicative of well folded proteins having predominantly \( \beta \)-sheet structure (minimum ellipticity at 215 nm) by far UV circular dichroism spectroscopy (Fig. 3B). Both constructs were stable against thermal denaturation up to at least 45 °C.

Fig. 4 shows titration calorimetry measurements for SB-247464 in the presence of \( \text{ZnCl}_2 \) alone and in the presence of \( \text{ZnCl}_2 \) plus the G-CSFR-Fc construct. As shown in Fig. 4A, binding of SB-247464 to \( \text{ZnCl}_2 \) is very tight (apparent \( K_d < 50 \) nM) and readily detected from the observed binding enthalpy change of the overall reaction. The molar ratio of \( 1:1 \) is consistent with preliminary x-ray crystallographic studies of the complexation of SB-247464 with metal ions, which showed formation of a quaternary complex consisting of two molecules of SB-247464 wrapped around two metal ions. To detect binding of SB-247464 to the receptor we compared the titrations of SB-247464 into zinc both in the presence and absence of G-CSFR-Fc construct. As seen in Fig. 4B, titration in the presence of G-CSFR-Fc resulted in a more exothermic process, with binding heats several kcal/mol higher than those obtained in its absence. The apparent \( K_d \) of the process was also higher (\( \sim 1-2 \) \( \mu \)M), indicating lower affinity of the SB-247464-zinc complex for the construct, compared with the binding of SB-247464 and zinc. Because of the presence of multiple equilibria, the apparent \( K_d \) derived from these data does not represent the true \( K_d \) for binding of the SB-247464-zinc complex to G-CSFR-Fc (see “Experimental Procedures”). Control experiments, either with the isolated Fc domain or with other Fc fusion proteins, demonstrated that the binding thermochromy observed with G-CSFR-Fc is due to interactions with the GCSFR-R portion of the construct. In the absence of zinc (Fig. 4C), binding of SB-247464 to G-CSFR-Fc could not be detected, indicating that, as in the reporter-gene experiments above, the interaction is mediated by zinc ions.

Evidence that SB-247464 Promotes Receptor Oligomerization—The presumed mechanism for activating G-CSFR receptor on the cell surface is induced self-association (9, 19–21). To further investigate the binding and agonist mechanism of SB-247464, we conducted analytical ultracentrifugation experiments on monomeric forms of the N- and C-terminal three-domain fragments of the receptor shown in Fig. 3. Fig. 5A shows analytical ultracentrifugation data for both constructs in the presence and absence of SB-247464; \( \text{ZnCl}_2 \) was used in these experiments. In the IgCk1Ck2 case (left), the addition of SB-247464 changed the shape of the sedimentation curve and increased the weight average mass from 46 to 66 kDa. In the FnFnFn case (right), the curves without and with SB-247464 had the same shape, differing only in amplitude, and the weight average masses were within error unchanged (54 versus 51 kDa, respectively). These mass-average molecular weights for FnFnFn are slightly higher than those determined by mass spectrometry. The difference may arise from uncertainties in the partial specific volume of the protein, the solution density, and potential deviations from the single exponential model used for curve fitting in Fig. 5A. Curve-fitting the FnFnFn data in Fig. 5A to an oligomerization model did yield an improvement in goodness of fit.

The residuals for the single-species fit (Equation 1) to the IgCk1Ck2 data were random in the absence of SB-247464 but showed a systematic trend in its presence, indicating lack of goodness of fit and hence the presence of multiple species. In contrast, SB-247464 had no discernible effect on the pattern of residuals for the FnFnFn fits.

The IgCk1Ck2 centrifuge data with SB-247464 can be inter-
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Fig. 4. SB-247464 binds to the extracellular domain of the murine G-CSF receptor in a zinc ion-dependent manner. Titration microcalorimetry data for mixing SB-247464 with 11 μM ZnCl₂ (A), 11 μM ZnCl₂ plus 5 μM murine G-CSFR-Fc (B), and 5 μM murine-G-CSFR-Fc plus 300 μM EDTA (C). Top panels, unprocessed microcalorimetry versus time data. At each injection of 10 μL of 150 μM SB-247464 into the calorimeter cell, a spike is observed. The integrated area of each spike yields the amount of heat for each titration increment. The lower panels show the amount of heat observed at each injection normalized per mole of SB-247464 injected versus the accumulated molar ratio of SB-247464 added per zinc ion. Curves are the best fit to an assumed single binding site model, with best-fit apparent parameters equal to $K_{app} = 0.047 \pm 0.020$ μM and $\Delta H_{app} = -11.9 \pm 0.2$ kcal/mol. Conditions were as follows: 10 mM HEPES, pH 7.3, 30°C, 150 mM NaCl, 30% sucrose, and 3% Me₂SO.

Fig. 5. SB-247464 changes the oligomerization state of the murine G-CSF receptor IgCk1Ck2 protein solution. A, analytical ultracentrifugation data of the effect of SB-247464 on the weight average molecular masses of murine IgCk1Ck2 (left) and FnFnFn (right) monomers. In each case, the data and best fit curves (Equation 1) are shown in the lower panels, and the residuals for the fits are shown in the upper panels. Open circles, in the presence of 10 μM SB-247464; solid squares, in its absence. Weight average masses of the constructs were determined as follows: 45.7 ± 0.4 kDa (IgCk1Ck2 alone), 66.3 ± 1.7 kDa (IgCk1Ck2 + SB-247464), 53.7 ± 0.1 kDa (FnFnFn alone), and 51.3 ± 0.1 kDa (FnFnFn + SB-247464). Conditions were as follows: 3 μM receptor protein, 15 μM ZnCl₂, 10 mM HEPES, pH 7.3, 20°C, 150 mM NaCl, 30% sucrose, 1% Me₂SO, and 20,000 rpm. B, interpretation of IgCk1Ck2 and SB-247464 centrifugation data with a monomer-dimer equilibrium model. Best-fit curve (Equation 2) is shown in the lower panel. The upper panel shows residuals for fitting to the monomer-dimer model (solid squares) together with residuals for a single-species model (Equation 1, open circles). Deconvolution of the fitted curve into predicted monomer (M) and dimer (D) species is shown as the dashed curves as labeled. The dotted line is the best fit absorbance offset (Equation 2).

reasonably fitted by a monomer-trimer model ($\chi^2 = 3.88 \times 10^{-3}$), but models proposing the existence of tetramers ($\chi^2 = 5.06 \times 10^{-3}$) or higher oligomers could be excluded as judged by lack of goodness of the fits. More thorough experimental testing of the monomer-dimer model by centrifugation was not carried out due to prohibitively long equilibration periods required in 30% sucrose solvent (3 days per condition). Overall, the data suggest that SB-247464 binds preferentially to an oligomeric form of IgCk1Ck2 relative to monomer, thus driving self-association of the receptor. This association was shown to be a noncovalent interaction, since analysis by nonreducing SDS-PAGE did not reveal higher molecular weight species expected from a disulfide cross-linking mechanism (data not shown).

A Nonsymmetrical Analogue of SB-247464 Acts as an Antagonist—The C₂-symmetrical structure of SB-247464 suggests that its capacity to dimerize the G-CSF receptor may result from its ability to recognize and bind to two receptor chains simultaneously, an interaction mediated by zinc ions. This model would predict that a derivative of SB-247464 with only single metal-binding and receptor recognition sites might be able to bind to, but not dimerize, the receptor and could potentially act as an antagonist of SB-247464. The imidazolinone SB-250017 (Fig. 1), with only one of the guanidinobenzimidazole groups of SB-247464, was identified as such a compound. Fig. 6A shows titration calorimetry data for SB-250017 binding to zinc (left) and zinc in the presence of G-CSFR-Fc (right). Here the binding enthalpy changes are smaller than with SB-247464, but the data readily demonstrate that SB-250017 can bind G-CSFR-Fc through an interaction also mediated by zinc(II). Analytical ultracentrifugation data demonstrated that SB-250017 did not induce self-association of the IgCk1Ck2 monomer (Fig. 6B).

Treatment of NFS60 cells with SB-247464 or G-CSF for 15 min causes activation of STAT3 and STAT5 that can be detected by electrophoretic mobility shift assay using a STAT-binding oligonucleotide (7, 28). This assay was used to test the effect of SB-250017 on the activation of G-CSF signal transduction pathways by SB-247464. NFS60 cells were incubated with 1 μM SB-247464 in the presence of either no SB-250017 or increasing concentrations of SB-250017. The medium was supplemented with 30 μM ZnCl₂ to provide an excess of zinc ions in the assay. SB-250017 alone did not cause activation of STATs, consistent with its inability to dimerize G-CSF receptor chains. However, SB-250017 significantly antagonized the activation of STATs by SB-247464 (Fig. 7). Increasing the concentration of ZnCl₂ in the assay to 100 μM did not relieve this repression, indicating that antagonism was not due to preferential chelation of zinc ions by SB-250017.
Internalization of G-CSF Receptors by SB-247464—Dimerization or oligomerization of receptors by cytokines and growth factors leads to internalization of receptors over a period of several hours (29). To seek additional evidence that SB-247464 affects the oligomerization state of G-CSF receptors in living cells, a receptor internalization assay was performed. NFS60 cells were preincubated with SB-247464 or G-CSF at 37°C, conditions that allow receptor internalization to occur (30–32). Following this preincubation, levels of residual G-CSF receptor at the cell surface were measured by staining with a fluorescently labeled G-CSF fusion protein. Fig. 8A shows that preincubation with SB-247464 at concentrations between 0.03 and 1.0 μM caused a reduction in cell surface G-CSF receptors compared with cells preincubated with medium alone. Interestingly, preincubation with higher concentrations of SB-247464 caused a smaller loss of cell surface receptors. As expected, preincubation with G-CSF caused a reduction in measured cell surface receptors, with a maximal effect occurring at 1 ng/ml. The assay was then performed at 4°C, a temperature that is nonpermissive for receptor internalization (30–32). Under these conditions, SB-247464 did not affect the ability of the fluorescent G-CSF fusion protein to bind to NFS60 cells, indicating that the reduction in cell staining seen at 37°C is not due to SB-247464 interfering with the binding of G-CSF to the receptor (Fig. 8B).

Additional Requirement for Transmembrane Sequences—Our previous work with chimeric human/murine G-CSF receptors had suggested that the C-terminal region of the murine extracellular domain appeared to be required for the activity of SB-247464 (23). This inference was based on the observation that a chimeric receptor in which the N-terminal half of the human G-CSF receptor extracellular domain was replaced with the equivalent region of the murine receptor was not responsive to SB-247464. We constructed additional mouse/human G-CSF receptor chimeras to examine this requirement further. These constructs were transfected into HepG2 cells and tested for their ability to activate a STAT-responsive reporter after G-CSF or SB-247464 treatment. The results are shown in Fig. 9. As previously reported (23), the murine G-CSF receptor is activated by SB-247464, whereas the human receptor is not. A
chimeric receptor in which the extracellular domain and first 11 amino acids of the transmembrane domain are murine in origin is activated efficiently by both G-CSF and SB-247464. However, a chimeric receptor in which the junction between murine and human sequences is at the start of the predicted transmembrane domain is only marginally activated by SB-247464, although activation by G-CSF is unaffected. A third chimeric receptor in which all of the transmembrane domain and the first 9 amino acids of the extracellular domain are human behaves similarly.

**DISCUSSION**

SB-247464 was originally discovered by virtue of its ability to activate G-CSF signal transduction pathways and was subsequently found to be a selective mimic of G-CSF both in vitro and in vivo (23). These are novel activities for a low molecular weight, nonpeptidyl molecule, and it was therefore of considerable interest to determine both the molecular target for SB-247464 and its mechanism of action. The cell-based assay used to identify SB-247464 can in principle detect compounds acting at many points in the G-CSF signaling cascade (33). Our initial characterization of SB-247464 indicated a requirement for murine G-CSF receptor expression for compound activity. However, it was not established whether the receptor was in fact the direct target for SB-247464 or whether it played an indirect role in compound activity.

Here we show that SB-247464 interacts with soluble extracellular domain fragments derived from the murine G-CSF receptor using both isothermal calorimetry and analytical ultracentrifugation. This strongly suggests that the extracellular domain of the receptor is the molecular target for SB-247464 and its mechanism of action. The cell-based assay used to identify SB-247464 can in principle detect compounds acting at many points in the G-CSF signaling cascade (33). Our initial characterization of SB-247464 indicated a requirement for murine G-CSF receptor expression for compound activity. However, it was not established whether the receptor was in fact the direct target for SB-247464 or whether it played an indirect role in compound activity.

Here we show that SB-247464 interacts with soluble extracellular domain fragments derived from the murine G-CSF receptor using both isothermal calorimetry and analytical ultracentrifugation. This strongly suggests that the extracellular domain of the receptor is the molecular target for SB-247464. The primary binding site for SB-247464 maps to the N-terminal half of the extracellular domain, comprising the Ig-like domain and the two FN-III-like domains of the CRH region. This is the same fragment of the receptor that binds to G-CSF (17, 18, 22). However, SB-247464 appears to be unable to compete with G-CSF for binding to the intact G-CSF receptor on NFS60 cells, suggesting that it binds to this region of the receptor at a site not involved in interactions with G-CSF. This is in contrast to the peptide mimics of erythropoietin and thrombopoietin and a recently described nonpeptidyl mimic of erythropoietin, which compete with the natural cytokine for receptor binding (34–36).

The in vitro binding studies show that SB-247464 does not bind to receptor in the absence of zinc ions. This explains the requirement for zinc ions for compound activity in the cell-based luciferase assay. Serum contained in the tissue culture media contains sufficient zinc ions to promote compound activity, but in biochemical assays it must be supplied exogenously. Isothermal calorimetry provides direct evidence that SB-247464 binds zinc ions, presumably via the two metal chelation sites present in the compound. The precise role of zinc ions in promoting compound binding is not known. In addition...
to their role in catalysis and stabilizing protein structure, zinc ions are known to mediate certain protein-protein interactions via coordination of amino acids in both proteins (37–39). In this case, the zinc ion may be coordinated to both SB-247464 and amino acids in the receptor, directly mediating compound binding. Alternately, zinc ions may indirectly promote compound binding by stabilizing the conformation or oligomerization state of SB-247464 that constitutes the receptor binding species.

Analysis of mouse/human chimeric G-CSF receptor constructs shows that amino acids in the murine transmembrane domain are required (but not sufficient) for efficient activation of the receptor by SB-247464. SB-247464 does not efficiently activate chimeric G-CSF receptors in which the transmembrane domain is entirely human in origin, even if they contain the binding site for the compound. The murine transmembrane domain may transmit or permit a conformational change in response to compound that is impeded by the human receptor transmembrane domain. In contrast, the response of chimeric G-CSF receptors to G-CSF is not influenced by the species derivation of the transmembrane domain.

The analytical ultracentrifuge experiments demonstrate that SB-247464 causes oligomerization of the G-CSF receptor extracellular domain. This observation provides an explanation for the ability of the compound to mimic the activities G-CSF in cellular and in vivo assays. Dimerization of the G-CSF receptor has been shown to be sufficient to trigger signal transduction, an observation that is generally true for homodimeric receptors of this family (40–45). Dimerization or higher order oligomerization of G-CSF receptor extracellular domain fragments in the presence of G-CSF has been reported in a number of biochemical studies and is evident in the crystal structure of the receptor CRH domain bound to G-CSF (17–21). The symmetry of SB-247464 allows the molecule to simultaneously bind to two zinc ions in a C2-symmetrical fashion. This property of SB-247464 suggests a model where the resulting SB-247464(Zn)2 ternary complex interacts with two different receptor chains, promoting their dimerization on the surface of the cell. This model is consistent with the observation that the related compound SB-250017, which lacks the symmetry elements of SB-247464 and contains a single zinc-binding site, can bind to the receptor but cannot dimerize it. Instead, SB-250017 acts as an antagonist of SB-247464, although it has no effect on the activation of receptor by G-CSF. This latter observation also suggests that the details of receptor dimerization by SB-247464 and G-CSF differ, perhaps indicating that there are multiple pathways that lead to receptor activation.

Internalization of G-CSF receptors by SB-247464 provides additional evidence that this compound induces receptor clustering. The shape of the dose-response curve in this assay warrants discussion. Increasing concentrations of SB-247464 initially induce greater levels of receptor down-regulation, up to 1 μM. Higher concentrations reduce receptor internalization, however. This inverted bell-shaped dose-response curve mirrors the bell-shaped dose-response curve seen with SB-247464 in the luciferase assay. Increasing concentrations of SB-247464 initially cause increased levels of luciferase expression, but higher concentrations elicit lower levels of luciferase expression. This effect is not due to compound toxicity. If SB-247464 induces receptor dimerization by binding to two receptor chains, these dose-response curves can be explained by self-antagonism of the compound at high concentrations due to occupation of all available receptor chains by a single molecule of SB-247464. A similar phenomenon has been described for growth hormone and erythropoietin (42, 43) but has not been observed for G-CSF (46), again suggesting that SB-247464 activates the receptor in a novel manner. A detailed review of this effect and its implications has been presented by Whitty and Borysenko (47).

Our data do not exclude an alternate model for SB-247464-mediated receptor dimerization, in which the compound binds to a single receptor chain and induces a conformational change that favors dimerization. However, this model does not readily explain the dose-response curves described above. Structural data showing the details of SB-247464 interaction with the receptor will allow a more complete understanding of the mechanism of action of this compound and may provide insight into differences between G-CSF- and SB-247464-mediated receptor activation.

These results show that low molecular weight compounds can selectively bind, dimerize, and activate relatively large receptors that are normally activated by protein ligands. Since compounds such as SB-247464 are readily amenable to optimization through standard medicinal chemistry techniques, it should be possible to develop small molecule, orally bioavailable drugs with the potential to replace injectable recombinant proteins such as cytokines.

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Selective Binding and Oligomerization of the Murine Granulocyte Colony-stimulating Factor Receptor by a Low Molecular Weight, Nonpeptidyl Ligand

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