A Small, Nonpeptidyl Mimic of Granulocyte–Colony-Stimulating Factor

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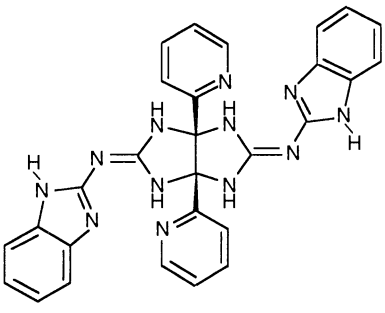
A nonpeptidyl small molecule SB 247464, capable of activating granulocyte–colony-stimulating factor (G-CSF) signal transduction pathways, was identified in a high-throughput assay in cultured cells. Like G-CSF, SB 247464 induced tyrosine phosphorylation of multiple signaling proteins and stimulated primary murine bone marrow cells to form granulocytic colonies in vitro. It also elevated peripheral blood neutrophil counts in mice. The extracellular domain of the murine G-CSF receptor was required for the activity of SB 247464, suggesting that the compound acts by oligomerizing receptor chains. The results indicate that a small molecule can activate a receptor that normally binds a relatively large protein ligand.

Activation of transmembrane receptors for growth factors and cytokines occurs when oligomerization of receptor chains is triggered by binding of a protein ligand to a specific ligand-binding domain on the receptor (*1, 2*). The resultant clustering of tyrosine kinase domains on the cytoplasmic side of the receptor initiates a series of signal transduction events that ultimately alter cellular phenotype. Receptors can also be activated by bivalent receptor antibodies (*2, 3*) and by dimeric peptides that interact with the ligand binding domain (*4*), which also induce receptor oligomerization. Activation of receptors by small, nonpeptidyl molecules amenable to chemical synthesis would make possible the development of orally available growth factor and cytokine mimics.

The protein hormone granulocyte–colony-stimulating factor (G-CSF) has a primary role in the production and activation of cells of the granulocytic lineage (*5*). Recombinant G-CSF is used to treat a variety of congenital and iatrogenic human neutropenias (*6*). Binding of G-CSF to its receptor triggers receptor homodimerization, which leads to activation of two members of the JAK family of protein tyrosine kinases, JAK1 and JAK2, which associate with the cytoplasmic domain of the receptor (*7, 8*). The activated JAKs phosphorylate tyrosine residues on the cytoplasmic face of the receptor, which then serve as the binding sites for signaling proteins. The JAKs are then presumed to phosphorylate the receptor-associated proteins, among which are the STATs (signal transducers and activators of transcription). After phosphorylation on tyrosine, the STATs dimerize, translocate to the nucleus, and bind to specific DNA sequences in the promoters of responsive genes, thereby regulating transcription (*7, 9, 10*).

We developed a high-throughput, cell-based screen to detect compounds that activate the G-CSF receptor. The screen relies on a reporter gene driven by a synthetic STAT-responsive promoter that is stably transfected into a G-CSF–responsive cell line. We isolated a drug-resistant clone of the murine myeloid cell line NFS60 that contained a G-CSF–responsive reporter construct consisting of four copies of a synthetic STAT-binding element linked to a minimal promoter and the gene for luciferase (*11*). This clone, 4B6, exhibited a 20-fold increase in luciferase activity in response to G-CSF and a pattern of JAK and STAT activation similar to that seen in the parental NFS60 cells (*9*). For screening, 4B6 cells were exposed to individual synthetic organic compounds at a concentration of 10 μM, and one compound, SB 247464 (Fig. 1), was selected for further study. In the luciferase assay, SB 247464 (1 μM) had an efficacy 30% of that of G-CSF and exhibited a biphasic dose-response curve (Fig. 2). The activity of SB 247464 was evaluated on a second NFS60-based stable cell line, RSVluc, which contains stably integrated copies of a reporter plasmid that produces luciferase constitutively (*12*). The level of luciferase activity in this line is not affected by G-CSF or SB 247464 (*13*). Likewise, SB 247464 had no effect in stable cell lines containing STAT-responsive reporters that increase luciferase activity in response to either erythropoietin, interferon α, or interferon γ (*13*).

We tested whether SB 247464 caused activation of signal transduction pathways normally activated by G-CSF. Proteins from lysates of NFS60 cells treated with SB 247464 or G-CSF were precipitated with antisera to JAK1, JAK2, G-CSF receptor, STAT3, or STAT5 and detected with an antibody to phosphotyrosine. Like G-CSF, SB 247464 caused tyrosine phosphorylation of both



SB 247464

Fig. 1. Structure of SB 247464. The benzimidazole groups are arbitrarily shown in the *trans* configuration.

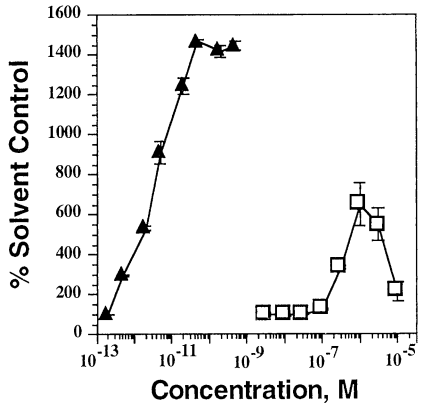


Fig. 2. Activity of G-CSF and SB 247464 in NFS60 cell luciferase assays. Dose-response curves are shown for G-CSF and SB 247464 in NFS60 cells containing a G-CSF–responsive reporter. Cytokine-independent NFS60 cells containing a stably integrated G-CSF–responsive luciferase reporter plasmid were plated in 96-well plates in Roswell Park Memorial Institute (RPMI) 1640 media containing fetal bovine serum (FBS) (0.5%), then treated with the indicated concentration of human G-CSF (▲) or SB 247464 (□) in the presence of 0.1% dimethyl sulfoxide (DMSO) for 2.5 hours. Cells were lysed, and luciferase activity was measured. All determinations were made in triplicate.

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JAK1 and JAK2 (Fig. 3A). SB 247464 also caused tyrosine phosphorylation of the G-CSF receptor, but not the interleukin-3 (IL-3) receptor (Fig. 3B). Both G-CSF and SB 247464 induced tyrosine phosphorylation of STAT3 and STAT5 (Fig. 3C). The two bands that became tyrosine phosphorylated in the STAT3 immunoprecipitations represent STAT3 isoforms (*14*). The time course of STAT activation in response to SB 247464 or G-CSF was very similar.

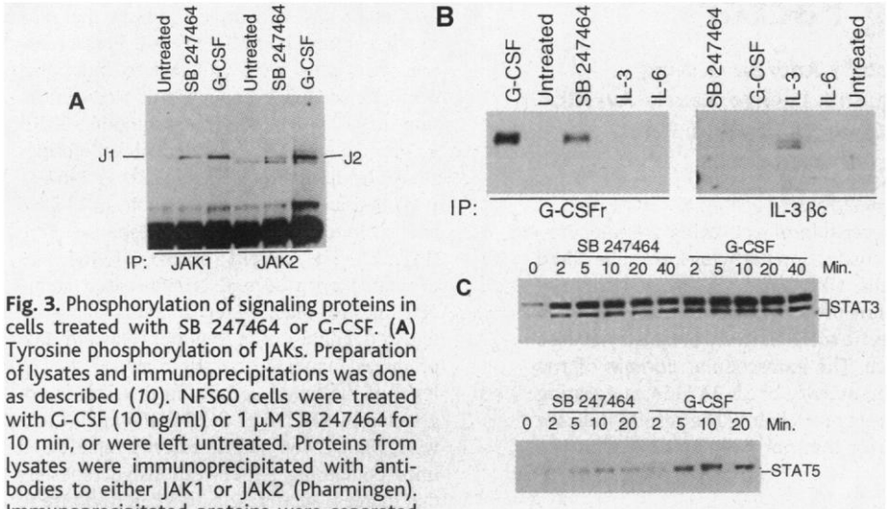

Fig. 3. Phosphorylation of signaling proteins in cells treated with SB 247464 or G-CSF. (**A**) Tyrosine phosphorylation of JAKs. Preparation of lysates and immunoprecipitations was done as described (*10*). NFS60 cells were treated with G-CSF (10 ng/ml) or 1 μM SB 247464 for 10 min, or were left untreated. Proteins from lysates were immunoprecipitated with antibodies to either JAK1 or JAK2 (Pharmingen). Immunoprecipitated proteins were separated by electrophoresis (8% gel), blotted onto a membrane, and detected with an antibody to phosphotyrosine (4G10, Upstate Biotech, Lake Placid, NY). (**B**) Tyrosine phosphorylation of the G-CSF receptor. NFS60 cells were treated for 10 min with G-CSF (10 ng/ml), IL-3 (10 ng/ml), IL-6 (10 ng/ml), or 1 μM SB 247464 in RPMI containing FBS (0.5%) and 0.1% DMSO. Lysates were made and processed as in (A), except that a polyclonal antisera to the cytoplasmic domain of the murine G-CSF receptor (*15*) or to the β-chain of the IL-3 receptor (Santa Cruz) was used for immunoprecipitations. (**C**) Tyrosine phosphorylation of STAT proteins. NFS60 cells in RPMI containing FBS (0.5%) were treated with G-CSF (10 ng/ml) or 1 μM SB 247464 for the indicated times, or were left untreated. Lysates were prepared and immunoprecipitated as described in (A) with antibodies to either STAT3 (J. Darnell, Upstate Biotechnology) or STAT5 (Santa Cruz).

We tested whether ectopic expression of the murine G-CSF receptor was sufficient to confer sensitivity to SB 247464 on nonresponsive cells. The human megakaryocytic cell line UT7Epo (*15*) does not express the G-CSF receptor, and STATs were not activated after G-CSF or SB 247464 treatment (Fig. 4A). However, UT7Epo cells stably transfected with an expression vector containing the murine G-CSF receptor cDNA (*10, 16*) became responsive to both G-CSF and SB 247464, demonstrated by the induction of STAT-DNA complexes (Fig. 4A). Transfection of the human hepatoma cell line HepG2 (*17*) with a murine G-CSF receptor expression vector also conferred sensitivity to either G-CSF or SB 247464, as measured using a STAT-responsive luciferase reporter (Fig. 4B).

In a number of human myeloid G-CSF–responsive cell lines, SB 247464 failed to show measurable activity as judged by induction of activated STATs or G-CSF early response genes (*13*). This is in contrast to the lack of mouse-human species specificity exhibited by G-CSF itself. We exploited the species specificity of SB 247464 to determine whether it requires the extracellular or intracellular domain of the murine G-CSF receptor for activity. A chimeric murine-human G-CSF receptor was constructed by replacing the sequences encoding the extracellular domain and part of the

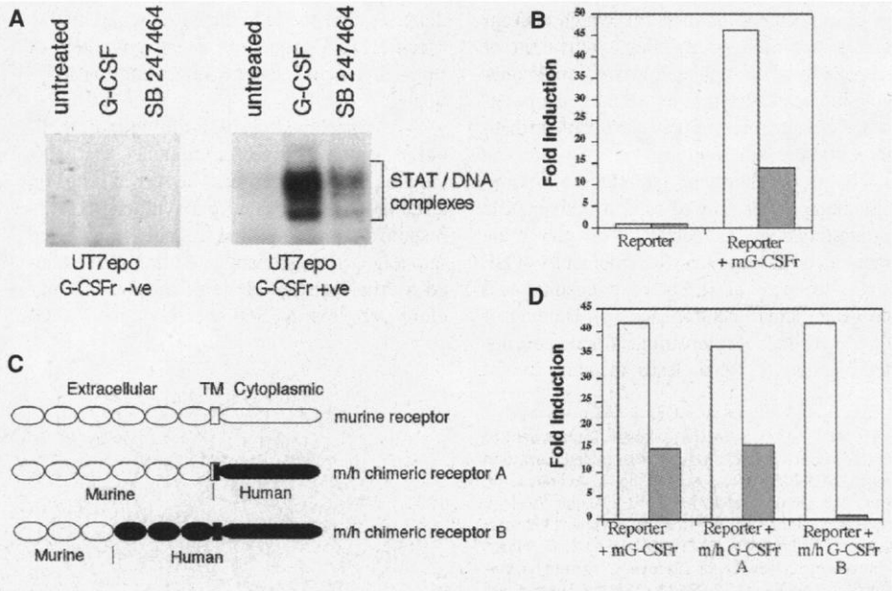

Fig. 4. The murine G-CSF receptor confers responsiveness to SB 247464. (**A**) UT7Epo cells (G-CSFr –ve) and UT7Epo cells stably transfected with the murine G-CSF receptor cDNA (G-CSFr +ve) were treated with G-CSF (10 ng/ml) or 1 μM SB 247464 for 30 min, or were left untreated. Nuclear extracts were prepared and incubated with a radiolabeled STAT-binding element, and STAT DNA complexes were separated from unbound DNA by nondenaturing gel electrophoresis. The gel was dried and exposed to x-ray film. (**B**) HepG2 cells were transfected with either 4xIRFtkluc (reporter) or with 4xIRFtkluc plus a vector directing the expression of the murine G-CSF receptor (*10, 16*) by the calcium phosphate method. Transfected cells were allowed to recover overnight, then were treated for 4 hours with either 10 ng/ml G-CSF (white bars) or 1 μM SB 247464 (gray bars). Control transfected cells were left untreated. Cells were then lysed, and luciferase levels were determined. Fold inductions were calculated by dividing the activity present in treated cells by that present in untreated cells. All transfections were performed in triplicate. (**C**) Domain structure of murine and chimeric G-CSF receptors. The location of the extracellular, cytoplasmic, and transmembrane (TM) domains are indicated. In the chimeric receptor, murine sequences are shown in white and human sequences are in black. The chimeric murine-human G-CSF receptor construct A was obtained by replacing the extracellular domain and the first 11 amino acids of the transmembrane domain of the human receptor with the Hind III (nucleotide 165) to Sca I (nucleotide 2087) fragment of the murine receptor(*16*). The chimeric receptor construct B was obtained by replacing the NH_2-terminal half of the extracellular domain of the human receptor (up to amino acid 339) with the Hind III (nucleotide 165) to Pml I (nucleotide 1199) fragment of the murine receptor. (**D**) The chimeric receptor constructs or the wild-type murine receptor construct were transfected into HepG2 cells with the 4xIRFtkluc reporter, treated, and processed as described in (B). Response to G-CSF is shown by the white bars; response to SB 247464 is shown by the gray bars.

transmembrane domain of the human receptor with the corresponding murine sequences (Fig. 4C). This chimeric construct was then transfected into HepG2 cells together with a STAT-responsive reporter. The chimeric receptor construct conferred a response to both G-CSF and SB 247464 on the HepG2 cells (Fig. 4D). This result implies that SB 247464 requires the extracellular domain of the murine G-CSF receptor for activity. A construct in which the NH_2-terminal half of the extracellular domain comprising the G-CSF binding region (*18*) is murine in origin, and the remainder of the receptor, which is human, did not confer responsiveness to SB 247464. This shows that the murine G-CSF receptor sequences required for SB 247464 activity are distinct from those required for G-CSF binding.

G-CSF normally acts on granulocytic precursor cells in the bone marrow, supporting their proliferation and differentiation. In a primary marrow colony-forming unit–granulocyte (CFU-G) assay (*19*), G-CSF and SB 247464 supported the formation of granulocytic colonies (Fig. 5). The peak efficacy of SB 247464 varied between 25 to 80% of that of G-CSF in different experiments. Colonies stimulated by SB 247464 appeared uniformly smaller than those stimulated by G-CSF, but were consistently larger than 30 cells.

Subcutaneous administration of 50 μg of G-CSF per kilogram of body weight twice a day to normal mice results in a fourfold increase in peripheral blood neutrophil counts after 4 days (Fig. 6). SB 247464 also caused a dose-dependent increase in peripheral blood neutrophils. No significant changes were noted in other blood cell populations. The efficacy of SB 247464 at 30 mg/kg is equivalent to that of 50 μg/kg of G-CSF, raising neutrophil counts approximately fourfold over baseline (Fig. 6). This fold-increase is equivalent to the increase seen when 5 to 30 μg/kg/day of G-CSF is administered to normal or neutropenic humans.

G-CSF, like other cytokines in the same family, acts by triggering dimerization or higher order oligomerization of its receptor chains (*2, 20*). The precise mechanism by which it does this is unclear, as is the mechanism by which SB 247464 is able to mimic the protein cytokine G-CSF. However, the fact that SB 247464 rapidly activates early events in the G-CSF signal transduction pathway, together with the ability of the transfected murine G-CSF receptor cDNA to confer both G-CSF and SB 247464 response to nonresponsive cells, shows that SB 247464 acts through the receptor. The twofold rotational symmetry of SB 247464 is compatible with a model in which it functions in some way as a ligand to effect dimerization of G-CSF receptor chains. This model of SB 247464 action would account for the biphasic dose response, as has been described for growth hormone (*2*). Although in longer term assays SB 247464 appears toxic at the highest concentrations used, this is not seen in short-term assays, indicating that the shape of the dose-response curve is not simply due to toxicity.

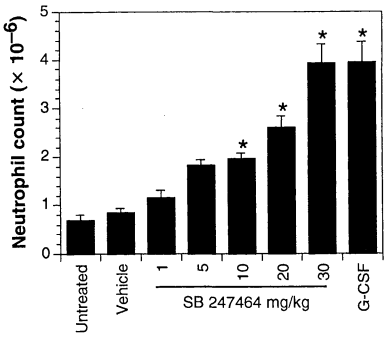

Fig. 6. Granulopoietic activity of SB 247464 in vivo. Female BDF-1 mice were given subcutaneous injections twice daily, of either G-CSF (50 μg/kg) in phosphate-buffered saline or SB 247464 dissolved in acidified H_2O (pH 4.0). Control animals received only acidified H_2O. After 4 days, blood was drawn and the number of neutrophils were counted using a Technicon hematology analyzer. Each bar represents the average of five mice; error bars show SEM. Asterisks indicate neutrophil counts that differ from those in untreated controls with a $P < 0.001$ by analysis of variance.

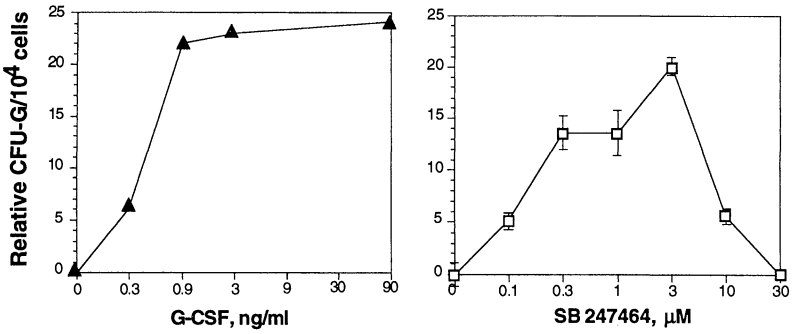

Fig. 5. Granulocytic colony formation in response to SB 247464 in vitro. Bone marrow cells (100,000 cells per 0.5 ml) obtained from female C57Bl/6 mice were incubated with either G-CSF or SB 247464 in McCoy's 5a media containing with FBS (15%) and 0.3% agar for 7 days at 37°C in a humidified incubator. Colonies of cells (>30 cells) were counted by microscopy. Data shown are the relative number of CFU-G colonies per 10^4 cells induced by each treatment. The relative number of colonies is the number of colonies in cultures of treated cells minus the number of colonies formed by control, untreated cells. Error bars indicate the standard error of the mean (SEM; $n = 6$).

The discovery of SB 247464 demonstrates that a small, nonpeptidyl molecule is capable of inducing activities normally associated with a protein hormone, both in vitro and in vivo. Our findings indicate that a small molecule can trigger the activation of a large (~120 kD) receptor protein that requires dimerization for activation, through a domain not involved in binding the natural ligand.

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11. IL-3 was withdrawn from NFS60 cells. A cytokine-independent variant of NFS60 grew out, which was used for transfection experiments. The G-CSF signaling characteristics of this line are similar to the parental NFS60s. This line was transfected by electroporation with the plasmid 4xTTCC1Ctkluc, which contains four copies of the STAT-binding element 5′-GATCTGCTTCCCGGAACGT-3′ cloned into the plasmid tk-luc (*21*), and the plasmid pSV2neo, which confers neomycin resistance. Single drug-resistant clones were isolated by limiting dilution and screened for increased luciferase expression after a 2.5-hour exposure to 10 ng/ml G-CSF.
12. The plasmid RSVluc, containing the Rous sarcoma virus long terminal repeat linked to luciferase, was transfected into the cytokine-independent NFS60 line with the plasmid pSV2neo conferring neomycin resistance. Single drug-resistant clones were isolated by limiting dilution and were screened for luciferase production before and after a 2.5-hour treatment with 10 ng/ml G-CSF. A clone showing no response to G-CSF was expanded.
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