Activity of the farnesyl protein transferase inhibitor SCH66336 against BCR/ABL-induced murine leukemia and primary cells from patients with chronic myeloid leukemia

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BCR/ABL, the oncoprotein responsible for chronic myeloid leukemia (CML), transforms hematopoietic cells through both Ras-dependent and -independent mechanisms. Farnesyl protein transferase inhibitors (FTIs) were designed to block mutant Ras signaling, but they also inhibit the growth of transformed cells with wild-type Ras, implying that other farnesylated targets contribute to FTI action. In the current study, the clinical candidate FTI SCH66336 was characterized for its ability to inhibit BCR/ABL transformation. When tested against BCR/ABL-BaF3 cells, a murine cell line that is leukemogenic in mice, SCH66336 potently inhibited soft agar colony formation, slowed proliferation, and sensitized cells to apoptotic stimuli. Quantification of activated guanosine triphosphate (GTP)-bound Ras protein and electrophoretic mobility shift assays for AP-1 DNA binding showed that Ras effector pathways are inhibited by SCH66336. However, SCH66336 was more inhibitory than dominant-negative Ras in assays of soft agar colony formation and cell proliferation, suggesting activity against targets other than Ras. Cell cycle analysis of BCR/ABL-BaF3 cells treated with SCH66336 revealed G2/M blockade, consistent with recent reports that centromeric proteins that regulate the G2/M checkpoint are critical farnesylated targets of FTI action. Mice injected intravenously with BCR/ABL-BaF3 cells developed acute leukemia and died within 4 weeks with massive splenomegaly, elevated white blood cell counts, and anemia. In contrast, nearly all mice treated with SCH66336 survived and have remained disease-free for more than a year. Furthermore, SCH66336 selectively inhibited the hematopoietic colony formation of primary human CML cells. As an oral, nontoxic compound with a mechanism of action distinct from that of ABL tyrosine kinase inhibition, FTI SCH66336 shows promise for the treatment of BCR/ABL-induced leukemia. (Blood. 2001;97: 1404-1412)

Introduction

Nearly 95% of patients with chronic myeloid leukemia (CML) and 20% of patients with acute lymphocytic leukemia (ALL) are Philadelphia chromosome (Ph1)-positive. Studies in murine models have implicated BCR/ABL, the chimeric oncogene product of the Philadelphia chromosome, as the primary causative factor in these diseases.[1-3] The 210-kd oncoprotein product of BCR/ABL (P210) observed in most cases is a nonreceptor tyrosine kinase that possesses greater enzymatic activity than its normal c-Abl counterpart. BCR/ABL induces leukemia by a complex process that includes inappropriate activation of cytokine receptor signaling pathways, altered adhesion properties of hematopoietic progenitors in the bone marrow, enhanced cycling of hematopoietic precursor cells, and decreased apoptosis of mature cells.[4]

CML arises in a Ph1-positive hematopoietic stem cell, and, in the early stages of the disease, the clinical syndrome is dominated by granulocytosis and splenomegaly owing to the accumulation of excessive numbers of well-differentiated myeloid cells. Patients in this benign "chronic" phase typically respond to treatment with antiproliferative chemotherapeutic agents such as busulfan or hydroxyurea and with interferon-α, which acts through both antiproliferative and immune mechanisms. Despite treatment, the chronic phase invariably progresses to terminal "blast" crisis, as reflected by the massive accumulation of immature myeloblasts or lymphoblasts in the circulation. This terminal phase of CML blast crisis resembles an aggressive acute leukemia, and the disease is rapidly fatal. The only curative treatment for CML is allogeneic bone marrow transplantation, which is available to only a small number of suitable candidates. Limitations in CML treatment make identification of novel therapies an important goal. Toward this end, emphasis has been placed on designing and testing novel compounds that inhibit the ABL tyrosine kinase and downstream signaling pathways activated by BCR/ABL. Small-molecule–substrate mimics called tyrphostins have been under development for many years.[5,6] Most recently, a novel structural entity, CGP57148B or STI571 (Novartis, Basel, Switzerland), has shown promise in murine models and early-phase human clinical trials.[7] However, the isolation of Ph1-positive human cell lines that are resistant to STI571[8,9] and the emergence of STI571 resistance in patients with acute forms of Ph1-positive leukemia highlight the

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need for combination chemotherapy to eradicate disease. Therefore, targeting other pathways in BCR/ABL-transformed cells should provide enhanced activity of chemotherapy in this disease and demonstrate proof of principle for pathway inhibition that might be effective against a variety of leukemias.

Genetic and biochemical data argue that Ras activation plays a central role in leukemogenic transformation by BCR/ABL. BCR/ABL couples to the Ras pathway through several protein-protein interactions with components of the Ras–MAP kinase signaling complex, including Grb2, SHC, and Crk-L.[10-14] Inhibition of Ras signaling by expression of dominant-negative Ras, overexpression of the catalytic domain of the Ras effector RasGAP, blockade of Grb2 adaptor protein function, or incubation with antisense oligonucleotides to p21Ras blocks BCR/ABL transformation in several cell culture models.[15-18]

Direct mutational activation of Ras and constitutive activation of the Ras signaling pathway are common themes in human cancer. Ras function depends on proper subcellular localization to the plasma membrane, which is facilitated in part by the addition of a 15-carbon isoprenoid moiety to the carboxy terminus, a reaction catalyzed by the farnesyl protein transferase (FPT) enzyme. Farnesyl protein transferase inhibitors (FTIs) are a class of drugs designed to specifically block oncogenic Ras signaling and Ras-dependent cellular transformation.[19] Blockade of FPT by FTIs disrupts Ras prenylation; without proper subcellular localization, mutant Ras is no longer oncogenic.[20] The rational design of small molecule inhibitors of the FPT has yielded compounds that are remarkably specific for FPT inhibition and relatively nontoxic to normal biologic functions, even at therapeutically effective concentrations.[21] Numerous studies have established the potent antitumor activity of FTIs against Ras-transformed murine and human cancer cells in vitro and Ras-specific tumor formation in transgenic and xenograft murine models.[22,23] These compounds have also proven effective at inhibiting the growth of transformed cells that lack mutant Ras,[24-26] suggesting that the blockade of other farnesylated targets may be important for the mechanism of action of this class of compounds. Recently, a survey of the database for potential farnesylated protein candidates that harbor a CAAX motif revealed several members of a class of centromere-associated proteins that act as mitotic kinesins, including CENP-E and CENP-F.[27] Incubation of cells with FTI blocks farnesylation of CENP-E, alters localization of CENP-E to microtubules, and arrests cells before metaphase, consistent with the role of CENP-E as a critical substrate of FTI action.[27] In the presence of inhibitory doses of FTIs, some protein substrates become alternatively prenylated by the geranyl-geranyl protein transferase, and evidence suggests that an alternatively prenylated form of RhoB exerts anti-proliferative effects on transformed cells.[28,29] Thus, FTIs appear to have multiple potential targets of action besides Ras to account for antiproliferative activity in transformed cells.

The involvement of Ras-dependent mechanisms in BCR/ABL oncogenicity suggested that FTIs might be active agents in the treatment of BCR/ABL-induced leukemia. Therefore, we have tested a potent, nonpeptidic, small-molecule inhibitor of the farnesyl protein transferase, SCH66336, and demonstrated activity in vitro and in vivo against a murine model of BCR/ABL-induced leukemia. We also demonstrated the ability of SCH66336 to inhibit colony formation of cells from patients with CML. Our results show that FTI compounds are highly effective as single agents against BCR/ABL-transformed hematopoietic cells, and they identify FTIs as promising clinical candidates for the treatment of BCR/ABL-induced leukemia.

Materials and methods

Farnesyl protein transferase inhibitor compound

The compound used in this study, SCH66336, is a nonpeptidic, tricyclic, competitive antagonist of the FPT developed and manufactured by Schering-Plough (FTI; Kenilworth, NJ).[30] SCH66336 potently inhibits farnesylation of Ras substrates.[26,31] This compound has been described in detail elsewhere.[32-34] SCH66336 was received in lyophilized form, reconstituted in dimethyl sulfoxide (DMSO), and maintained at −20°C. For experiments in tissue culture, SCH66336 was diluted at least 1:500 in appropriate tissue culture media before it was added to in vitro assays. For oral treatment of mice, SCH66336 was reconstituted in 20% hydroxy-propyl-beta-cyclodextrin vehicle (HPβCD; Sigma, St Louis, MO) and maintained at 4°C. Control mice were treated with vehicle alone.

Cell lines for in vitro and in vivo analysis

BCR/ABL-mediated transformation of the interleukin-3 (IL-3)–dependent pro-B cell line BaF3[35] has been described previously.[36] Stable transformants (BCR/ABL-BaF3) were maintained in RPMI supplemented with 10% fetal calf serum, 2 mM glutamine, and 50 U/mL penicillin and streptomycin. Parental nontransformed BaF3 cells were grown in the same medium supplemented with 10% conditioned medium from the WEHI-3B cell line as a source of IL-3.

Colony formation in soft agar

To determine the effect of SCH66336 on the ability of BCR/ABL-BaF3 cells to form macroscopic colonies in soft agar, 10 000 hematopoietic cells were plated in each 3.5-cm well of a 6-well dish in appropriate growth media supplemented with 0.3% bacto-agar. Media were supplemented with SCH66336 at the range of concentrations specified. Macroscopic colonies were counted in duplicate plates on day 10. Colony numbers were normalized by dividing the number of colonies under a given condition by the number of colonies formed in the absence of SCH66336.

Proliferation assay in liquid culture

To determine the effect of SCH66336 on the proliferation of BCR/ABL-BaF3 cells in liquid culture, 50 μL of a 2×10^5 cells/mL dilution was apportioned into separate wells of a 96-well plate (10 000 cells/well). Then 50 μL medium containing SCH66336 was added to obtain the range of concentrations specified. Proliferation rates were obtained at 24, 48, 72, and 96 hours using the WST-1 cell proliferation reagent as specified by the manufacturer (Roche Molecular Biochemicals, Indianapolis, IN). Absorbance was measured using an Analyst AD microtiter plate reader and Criterion Host software (LJL Biosystems, Sunnyvale, CA) at 450-nm wavelength.

Cell cycle analysis and identification of apoptotic cells

Cell cycle analysis was performed on BCR/ABL-BaF3 cells growing in 0, 1, and 10 μM SCH66336 for 48 hours. Briefly, 5×10^6 cells were fixed in cold ethanol overnight. Cells were then collected, washed in phosphate-buffered saline (PBS)/1% bovine serum albumin (BSA), and resuspended in PBS/1% BSA. Propidium iodide was added to a final concentration of 0.05 mg/mL. RNase A was added, and digestion of RNA was carried out at 37°C for 30 minutes. Cell cycle analysis was then performed by flow cytometry using the Modfit LT program (Verity Software House, Topsham, ME). The apoptotic index for BCR/ABL-BaF3 cells after growth in SCH66336 was assessed by staining for annexin-positive cells using the ApoAlert Annexin V kit (Clontech, Palo Alto, CA) essentially as recommended by the manufacturer. Flow cytometric analysis was performed using Cell Quest software (Becton Dickinson, Franklin Lakes, NJ).

Detection of Ras-GTP using GST-Raf1 Ras-binding domain

FTI inhibition of Ras activity was demonstrated by the precipitation of Ras-GTP using the Ras-binding domain (RBD) of Raf1 (aa51-131), as described previously.[37] The vector pGEX 2T-RBD, which encodes the GST-Raf1 RBD fusion protein, was a kind gift of J. L. Bos. Bacterial lysates containing GST-Raf1 RBD were incubated with GST-bind Resin (Novagen) for 1 hour at 4°C. GST-coupled resin was isolated by centrifugation and washed 5 times with NP-40 lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM Na_3VO_4, 1 mM $MgCl_2$, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol). Whole-cell lysates of BCR/ABL-BaF3 cells that were grown in the presence of 0.25 to 1 μM SCH66336 or 0.01% DMSO (control) for 48 hours were prepared by lysis in NP-40 lysis buffer. GST-coupled resin was added to approximately 1 mg whole cell extract and incubated for 1.5 hours at 4°C. Resin was then collected by centrifugation, washed 3 times with NP-40 lysis buffer, and resuspended in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Protein samples were separated on a 15% SDS-PAGE gel and transferred to nitrocellulose membrane. Membranes were subjected to immunoblotting with an antibody specific to K-Ras (Santa Cruz Biotechnology, Santa Cruz, CA).

Electrophoretic mobility shift assay analysis of AP-1

BCR/ABL-BaF3 cells were grown in the presence of 0.5 μM SCH66336 or 0.01% DMSO (control) for 48 hours and then washed in cold PBS. Whole-cell extracts were prepared by resuspending cells in NP-40 lysis buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 10 mM Na_3VO_4, 1 mM $MgCl_2$, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 10% glycerol). Cellular debris was removed by centrifugation at 16 000*g*. Protein extracts were immediately frozen at −85°C. Complementary oligonucleotides that contained an AP-1 consensus binding site (5′-CGCTTGATGACTCAGCCGGAA-3′) were annealed and radiolabeled with γ-^{32}P-[ATP] using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Approximately 25 000 cpm (0.2 ng) electrophoretic mobility shift assay probe was incubated with 20 μg whole-cell extract in 20 μL 10 mM HEPES, pH 7.9, 50 mM KCl, 0.2 mM dithiothreitol, 10% glycerol, 0.05% Nonidet P-40, 1 μg poly-dIdC (Sigma, St Louis, MO) for 20 minutes at room temperature. Resultant DNA-protein complexes were resolved on a 5% nondenaturing polyacrylamide gel followed by autoradiography.

Assessing inhibition of farnesylation in BCR/ABL-BaF3 cells treated with FTI

Inhibition of farnesylation in BCR/ABL-BaF3 cells treated with FTI was determined by analysis of a representative farnesylated protein, DnaJ. The BaF3 and BCR/ABL-BaF3 cells were grown in media containing SCH66336 at the indicated concentrations. Control cells were grown in 0.1% DMSO. After 60 hours the cells were harvested and washed once in ice-cold PBS. The cells were lysed in buffer (50 mM HEPES, pH 7.8, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol) containing 1.0% Triton X-100 and protease inhibitors. After protein determination, 10 μg each lysate was separated by gel electrophoresis on a 10% SDS-polyacrylamide gel under reducing conditions. The proteins were then blotted onto nitrocellulose. HDJ-2 (DnaJ) protein was detected using a murine monoclonal antibody, Clone KA2A5.6 (Neomarkers, Lab Vision, Fremont, CA).

Measurement of viability of BCR/ABL-BaF3 cells after treatment with SCH66336 in low serum and after gamma irradiation

BCR/ABL-BaF3 cells were seeded in 3.5-cm wells of a 6-well tissue culture plate at a density of 5×10^4 cells/mL of RPMI-1649 media. Media contained either 10% or 0.1% fetal calf serum that had been inactivated by heating. Cells were exposed to up to 500 nM FTI SCH66336 or 0.01% DMSO (control). In some cases treated and control cells were exposed to 500 cGy gamma irradiation using a cesium Cs 137 source (Gammacell-40). Viability of cells was determined daily by trypan blue exclusion.

Characterization of a murine model of BCR/ABL-induced acute lymphoblastic leukemia and treatment with FTI

All animal studies were carried out at the Whitehead Institute (Cambridge, MA) animal care facility with the approval of the Massachusetts Institute of Technology (MIT) Committee on Animal Care in accordance with MIT guidelines (protocol 95-028-3). A graded dosage series of BCR/ABL-BaF3 cells diluted in 0.1-mL PBS was injected intravenously into the lateral tail veins of young Balb/c mice (4-6 weeks old; Jackson Laboratory, Bar Harbor, ME). Mice were observed thereafter for signs of clinical deterioration and were killed at the first signs of morbidity (loss of more than 10% body weight, reduced body temperature, rigors, decreased activity, hunched posture, decreased grooming). For in vivo assessments of FTI compounds, mice were injected with 1 million BCR/ABL-BaF3 cells and then randomly assigned to either control (vehicle alone) or FTI treatment groups. FTI compound SCH66336 was dissolved in 20% (wt/vol) HPβCD vehicle and administered by oral gavage (0.1 mL) beginning on day 1 in a dose of 40 mg/kg twice daily (BID; total dose, 80 mg/kg per day). Controls were given 0.1 mL vehicle BID and showed no signs of impaired health or toxicity. Mice were treated for 32 days, after which treatment was discontinued.

Histopathologic analysis

Pathologic analysis was performed by the MIT Division of Comparative Medicine according to standard protocols on mice injected with BCR/ABL-BaF3 cells and receiving either vehicle alone or treatment with the FTI SCH66336. Histology sections were stained with hematoxylin and eosin and photographed at either 100 × or 400 × magnification.

Methylcellulose colony assays of human primary cells

Total bone marrow cells from healthy human subjects and $CD34^+$ bone marrow and peripheral blood cells from patients with CML were plated in methylcellulose containing human growth factors (IL-3, IL-6, stem cell factor, erythropoietin; Methocult GF H4434; Stem Cell Technologies, Vancouver, BC, Canada) with or without SCH66336 at increasing concentrations. Initial experiments were conducted to determine the optimal seeding density so that plates without SCH66336 contained between 100 and 150 colonies/35-mm Petri dish (2×10^5 to 5×10^5 cells/plate for whole bone marrow and 2×10^3 cells/plate for $CD34^+$ cells). Blinded colony counts were performed on duplicate plates on day 14 using an inverted microscope.

Results

SCH66336 inhibits colony formation in soft agar and proliferation in liquid culture of BCR/ABL-transformed BaF3 cells. BaF3 is a cytokine-dependent murine hematopoietic cell line that requires IL-3 for proliferation in vitro.[35,38] Expression of BCR/ABL in these cells abrogates their growth-factor dependence and generates transformed lines that form tumor nodules when injected subcutaneously into nude mice and aggressive leukemias when injected intravenously into syngeneic mice.[36,39,40] BCR/ABL-BaF3 cells will also form colonies in soft agar, which provides a quantitative in vitro assay for the activity of FTIs against these transformed hematopoietic cells. SCH66336 shows a dose-dependent inhibition of colony formation for the BCR/ABL-transformed BaF3 cells, with an IC_{50} (50% inhibitory concentration) of approximately 100 nM (Figure 1).

To assess the effect of SCH66336 on the proliferation rate of BCR/ABL-BaF3 cells in liquid culture, BCR/ABL-BaF3 cells were grown in increasing concentrations of SCH66336. There was a dose-dependent reduction in proliferation rate with an IC_{50} between 0.5 and 1 μM and complete inhibition of proliferation at approximately 10 μM (Figure 2). Expression of dominant-negative Ras had no significant effect on the proliferation rate of the

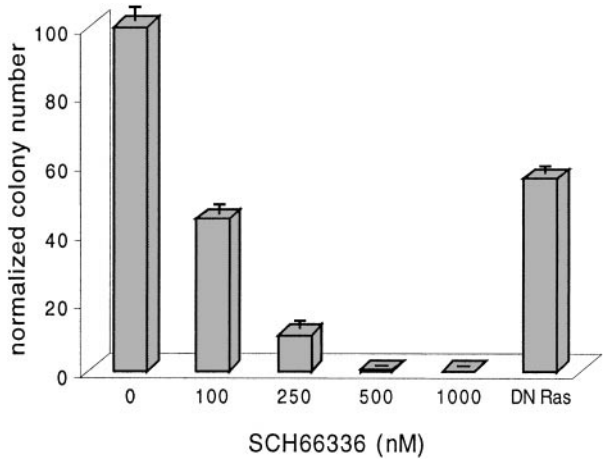

Figure 1. FTI SCH66336 inhibited colony formation of BCR/ABL-transformed BaF3 cells grown in soft agar. Graph shows colony formation in soft agar of BCR/ABL-BaF3 cells in the presence of indicated concentrations of SCH66336. IC_{50} is approximately 100 nM. Colony number is normalized to the number of colonies without SCH66336. Soft agar colony formation is also shown for BCR/ABL-BaF3 cells expressing a dominant-negative (DN) form of Ras.

BCR/ABL-BaF3 cells (not shown) while inhibiting colony formation in soft agar by nearly 50% (Figure 1). For unclear reasons, both the FTI compound and dominant-negative Ras are more potent at inhibiting soft agar colony formation than proliferation in liquid culture.

Cell cycle profile and apoptotic populations of BCR/ABL-BaF3 cells grown in SCH66336

To further investigate the biologic consequences of SCH66336 on BCR/ABL-BaF3 cells, the cell cycle and percentage of cells undergoing apoptosis were analyzed. BCR/ABL-BaF3 cells were grown in 0, 1, and 10 μM SCH66336 in liquid culture for 48 hours. The cell cycle profiles were then assessed by flow cytometry. BCR/ABL-BaF3 cells grown in 0 μM SCH66336 displayed a typical cell cycle profile (Figure 3A, top). Most of the cells exhibited intermediate DNA content (between 2N/4N), representing S phase, whereas some exhibited G2/M DNA content (4N). After growth in 1 μM SCH66336, a larger percentage of BCR/ABL-BaF3 cells existed in the G2/M stage, and there was a compensatory reduction in cells in S phase (Figure 3A, middle). Growth in 10 μM SCH66336 had an even greater effect on the cell cycle as BCR/ABL-BaF3 cells underwent a significant G2/M block (Figure 3A, bottom). The blockade of cell cycle progression responsible for the dramatic inhibition of proliferation of BCR/ABL-BaF3 cells appears to have resulted chiefly from failure to progress through mitosis.

Despite the profound inhibition of cell proliferation, FTI-treated BCR/ABL-BaF3 cells remained largely viable after 1 week in culture (not shown). Therefore, the effect of SCH66336 on BCR/ABL-BaF3 cell apoptosis was investigated by annexin staining. Cells grown in the absence of SCH66336 showed a modest degree of apoptosis with a population of annexin-positive, propidium iodide–negative cells of 1.2% (Figure 3B, top, lower-right quadrant). BCR/ABL-BaF3 cells grown in 1 μM SCH66336 showed a modest increase in the percentage of apoptotic cells (6.3%; Figure 3B, middle), which increased only slightly after growth in 10 μM SCH66336 (8.1%; Figure 3B, bottom). SCH66336 appeared to have its principal effect on the cell cycle, causing a relatively potent G2/M block, rather than on the induction of apoptosis.

FTI treatment sensitized BCR/ABL-transformed BaF3 cells to a second signal for apoptosis. Previous work suggests that the FTI SCH66336 may sensitize cells to apoptosis under conditions of added stress.[41] To determine whether SCH66336 sensitizes BCR/ABL-BaF3 cells to apoptotic stimuli such as serum withdrawal and gamma irradiation, the viability of FTI-treated BCR/ABL-BaF3 cells was assessed after growth in 10% or 0.1% serum and after exposure to gamma irradiation (500 cGy; Figure 4). SCH66336 (0.5 μM) had no effect on the viability of nonirradiated BCR/ABL-BaF3 cells grown in 10% serum. In contrast, SCH66336 treatment caused a considerable reduction in viability in BCR/ABL-BaF3 cells grown in 0.1% serum. SCH66336 also appears to have reduced the radioprotective effect of BCR/ABL transformation. Irradiated BCR/ABL-BaF3 cells grown in both 10% and 0.1% serum had significantly reduced viability when exposed to SCH66336. These results suggest that SCH66336 causes sensitization to apoptotic stimuli such as serum withdrawal and irradiation, consistent with previous reports.[41]

FTI blocks farnesylation of DnaJ in BCR/ABL-BaF3 cells. K-Ras, the predominant Ras isoform expressed in BaF3 cells, is subject to alternative prenylation by the geranyl-geranyl protein transferase under conditions of farnesyl protein transferase inhibition, and, therefore, blockade of farnesylation cannot be routinely assessed by alteration of its electrophoretic mobility[31] (data not shown). To assess the ability of SCH66336 treatment to inhibit cellular FPTase enzyme activity in BaF3 and BCR/ABL-BaF3 cells, a surrogate assay was performed on DnaJ, a protein that is not alternatively prenylated.[42] Treatment of BaF3 and BCR/ABL-BaF3 cells with SCH66336 results in the inhibition of DnaJ processing to the smaller, farnesylated form in both cell types (Figure 5C). Thus, FPTase activity is effectively inhibited by SCH66336 treatment in both BaF3 and BCR/ABL-BaF3 cells.

FTI treatment reduces levels of activated, GTP-bound Ras and

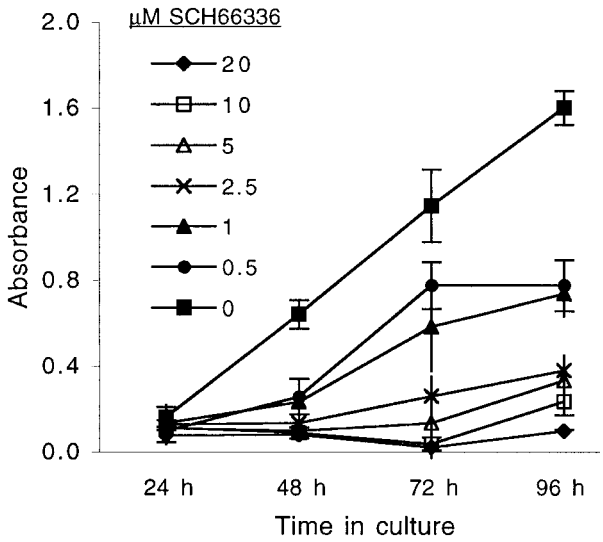

Figure 2. SCH66336 slowed the proliferation of BCR/ABL-BaF3 cells in liquid culture. BCR/ABL-BaF3 cells were split into medium containing the indicated concentration of SCH66336, and cell numbers were measured for 4 days. SCH66336 caused a dose-dependent reduction in proliferation with an IC_{50} of 0.5 to 1 μM. BCR/ABL-BaF3 cells expressing dominant-negative Ras show no perceptible reduction in proliferation (data not shown).

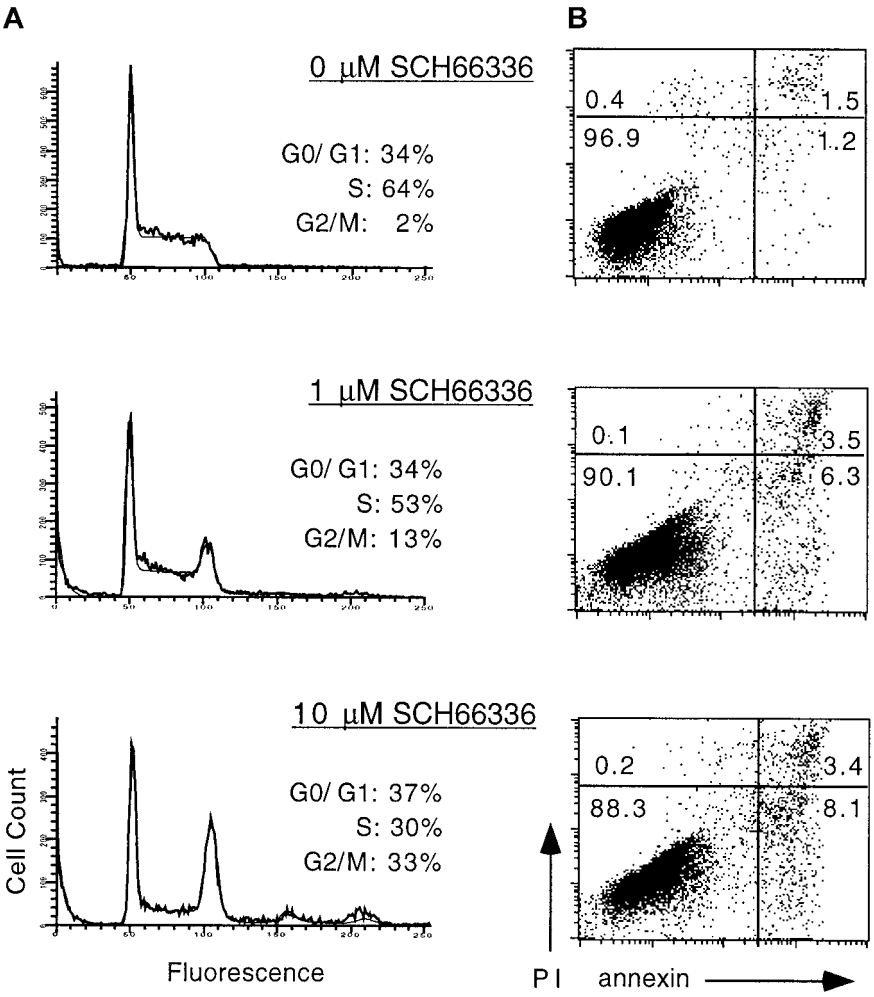

Figure 3. Cell cycle analysis and annexin staining of apoptotic cells from BCR/ABL-BaF3 cells grown in SCH66336. (A) BCR/ABL-BaF3 cells grown in medium containing 0, 1, or 10 μM SCH66336 were subjected to cell cycle analysis. Fixed cells were stained with propidium iodide and analyzed by flow cytometry. Increased concentrations of SCH66336 caused a reduction in the number of cells in S phase and an increase in G2/M. (B) BCR/ABL-BaF3 cells grown in medium containing 0, 1, or 10 μM were stained with propidium iodide to identify dead cells and annexin antibody to detect cells that had initiated apoptosis. Increasing concentrations of SCH66336 caused a modest increase in the number of cells that initiated apoptosis, as seen in the annexin-positive, propidium iodide–negative population (lower-right quadrant).

blocks AP-1 DNA binding in BCR/ABL-transformed cells. Because only the active, GTP-bound form of the Ras protein binds to its effector, Raf-1, the amount of activated Ras in a cell can be measured directly in a quantitative assay of binding to the effector domain of Raf-1 fused to GST.[37] BCR/ABL-BaF3 cells have high levels of Ras-GTP (Figure 5A). Levels of Ras-GTP were significantly reduced in BCR/ABL-BaF3 cells after treatment with 500 nM SCH66336, a concentration that maximally inhibited soft agar colony formation and modestly reduced the proliferation rate of BCR/ABL-BaF3 cells. By comparison, BCR/ABL-BaF3 cells expressing a dominant-negative Ras have no detectable levels of Ras-GTP.

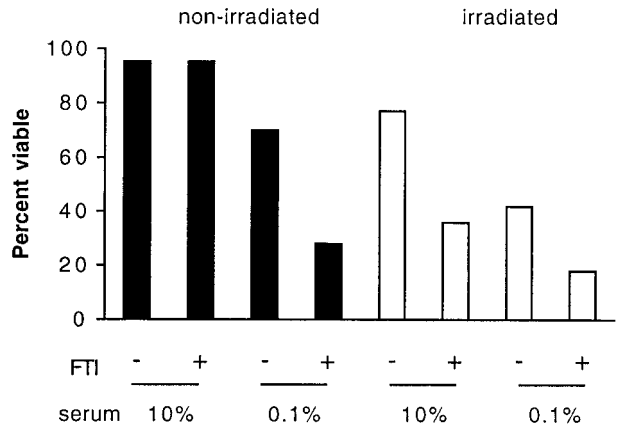

Figure 4. SCH66336 caused significant reductions in the viability of BCR/ABL-BaF3 cells grown in low serum and after irradiation. Viability of BCR/ABL-BaF3 cells was assessed by trypan blue exclusion after 48 hours of growth under the conditions specified. Black bars represent cells that were not exposed to gamma irradiation; white bars indicate those that were irradiated. Viability of BCR/ABL-BaF3 cells grown in 0.1% serum plus 500 nM SCH66336 was significantly reduced from that of BCR/ABL-BaF3 cells grown in 0.1% serum without SCH66336. After irradiation, SCH66336 treatment reduced viability in BCR/ABL-BaF3 cells grown in both 10% and 0.1% serum.

Activation of Ras/MAP kinase signaling leads to activation of the DNA binding activity of the AP-1 transcription factor complex.[43] We used an electrophoretic mobility shift assay to investigate the activation of AP-1 binding to its cognate DNA recognition sequence by the constitutively activated Ras mutant H-Ras-61L and by the BCR/ABL oncoprotein and to study the effect of treatment with FTI (Figure 5B). Incubation with IL-3 (lane 2) or transformation by H-Ras-61L (lane 3) or BCR/ABL (lane 4) lead to enhanced DNA binding by the AP-1 transcription factor. Co-expression with BCR/ABL of a dominant-negative form of H-Ras (17N) lead to marked inhibition of AP-1 DNA binding (lane 5), an effect mimicked by the incubation of BCR/ABL-transformed BaF3 cells with the FTI compound SCH66336 (lane 7). Incubation of cells transformed by H-Ras 61L with FTI also abrogated AP-1 binding (lanes 8,9). Our data argue that BCR/ABL activates the Ras/MAP kinase signaling pathway, leading to activation of the DNA binding activity of the AP-1 transcription factor complex, and that this effect of Ras signaling can be markedly inhibited by the FTI compound.

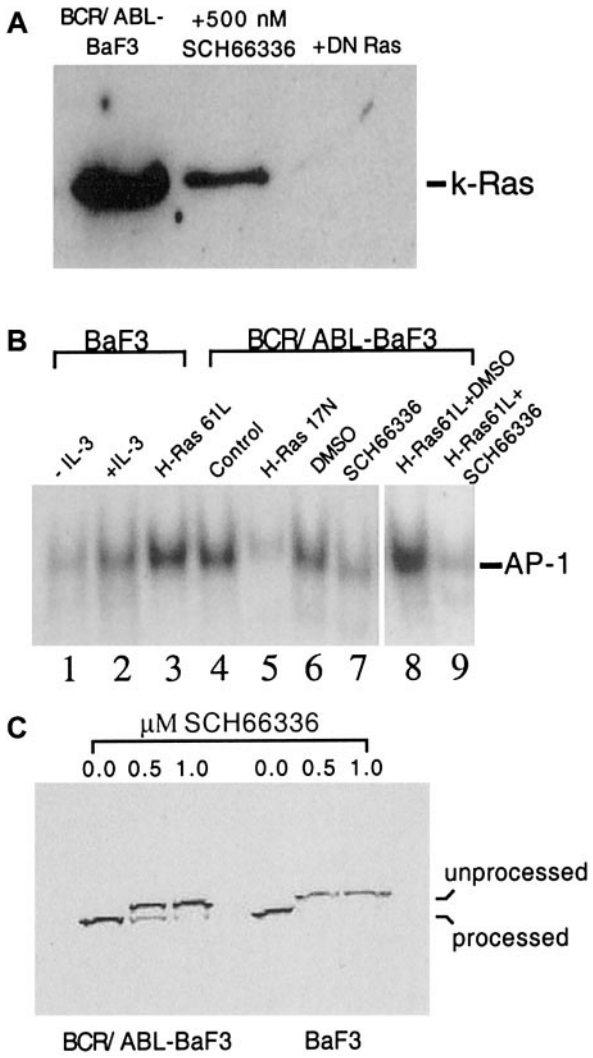

Figure 5. Assessment of Ras activity and farnesyl protein transferase inhibition in BCR/ABL-BaF3 cells treated with SCH66336. (A) Levels of activated, GTP-bound Ras protein in BCR/ABL-BaF3 cells was determined by quantifying the association with the effector domain of Raf-1 fused to GST. The high levels of active Ras-GTP protein in BCR/ABL-BaF3 cells were significantly reduced after treatment with 500 nM SCH66336. Expression of a dominant-negative (DN) form of Ras reduced the levels of Ras-GTP to undetectable levels. (B) DNA binding activity of AP-1 transcription factor complex in BaF3 and BCR/ABL-BaF3 cells as determined by electrophoretic mobility shift assay. Lanes 1 and 2: Parental BaF3 cells without IL-3 or treated with 25 ng/mL IL-3 for 30 minutes. Lane 3: BaF3 cells transduced with a retrovirus encoding an activated form of H-Ras (61L). Lane 4: BCR/ABL-BaF3 cells. Lanes 5-7: BCR/ABL-BaF3 cells transduced with a dominant-negative form of H-Ras (lane 5), treated with FTI vehicle DMSO (lane 6), or treated with 1 μM SCH66336 for 48 hours before extract preparation (lane 7). Lanes 8, 9: BaF3 cells transduced with a retrovirus encoding an activated form of H-Ras (61L) and treated with FTI vehicle DMSO (lane 8) or 1 μM SCH66336 (lane 9) for 48 hours before extract preparation. (C) Blot showing cellular inhibition of farnesylation by SCH66336 using a representative protein, DnaJ, which, unlike K-Ras, was not subject to alternative prenylation in the presence of FTI. BaF3 and BCR/ABL-BaF3 cells were incubated in the indicated concentrations of SCH66336, and DnaJ was detected by immunoblotting, as described in "Materials and methods." Farnesylation of DnaJ resulted in reduced molecular mass due to subsequent processing of the protein. Thus, the "unprocessed" band represents DnaJ that was not farnesylated, whereas the "processed" band represents DnaJ that was farnesylated. Farnesylation was almost completely inhibited in BCR/ABL-BaF3 cells treated with 1 μM SCH66336.

In vivo efficacy of FTI in a mouse model of BCR/ABL-induced leukemia

SCH66336 is a clinical candidate presently in trials against a variety of solid tumors but not yet tested against patients with leukemia. This compound was remarkably potent at inhibiting BCR/ABL-BaF3 growth in soft agar with an IC_{50} of approximately 100 nM and a maximal inhibition at 1 μM (Figure 1). Previous pharmacokinetic analysis in mice has demonstrated that single oral doses of 25 mg/kg SCH66336 achieve a C_{max} of 8.84 μM, with drug levels persisting above 700 nM for 9 hours.[26] Significant antitumor activity has been documented in murine models with simplified dosing regimens of 5, 20, and 80 mg/kg delivered BID.[26] Our preliminary experiments using an initial dose of 80 mg/kg BID resulted in more than 10% weight loss in our animals, whereas the 40 mg/kg BID regimen was well tolerated. This dosage is adequate for achieving sustained serum drug levels in vivo significantly above the concentrations that provide inhibition of soft agar colony formation for BCR/ABL-BaF3 cells and high enough to slow proliferation of BCR/ABL-BaF3 cells in liquid culture. Therefore, the favorable pharmacokinetics and bioavailability of this compound facilitated testing in a murine model of BCR/ABL-induced leukemia.

Intravenous injection of BCR/ABL-transformed BaF3 cells into syngeneic Balb/c mice results in an aggressive malignancy resembling acute leukemia, characterized by splenomegaly, circulating blasts, and invasion of leukemic cells into hematopoietic and

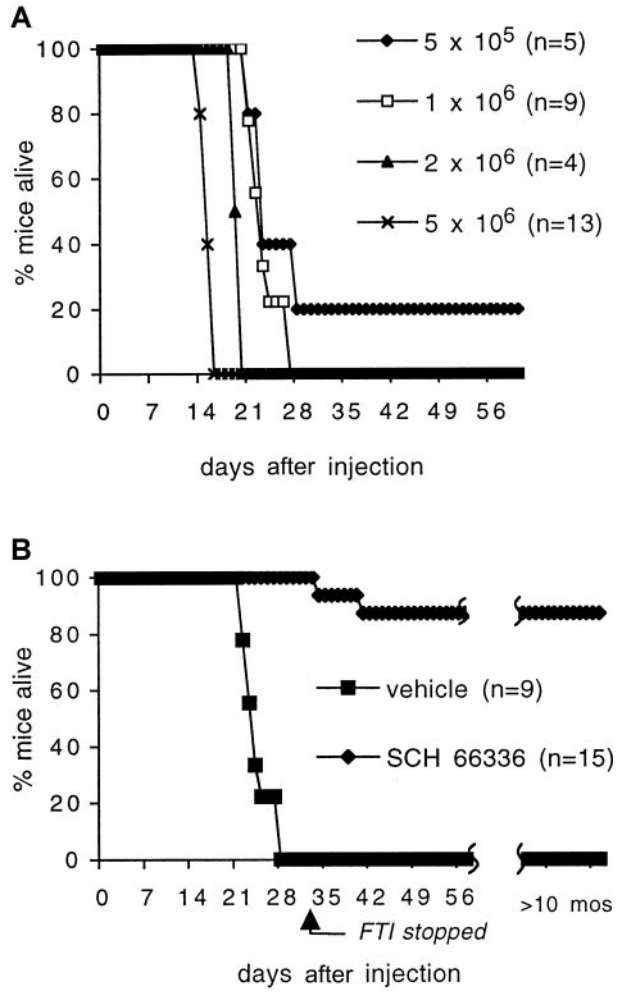

Figure 6. Activity of SCH66336 in a murine model of BCR/ABL-induced acute lymphoblastic leukemia. (A) Survival of mice injected with indicated doses of BCR/ABL-BaF3 cells. (B) Survival of mice injected with 10^6 BCR/ABL-BaF3 cells and treated with either vehicle alone or SCH66336. Treatment was stopped after 32 days. All mice that did not receive SCH66336 died of a condition resembling acute leukemia by 28 days; nearly all mice treated with SCH66336 survived for more than 1 year and remain disease-free.

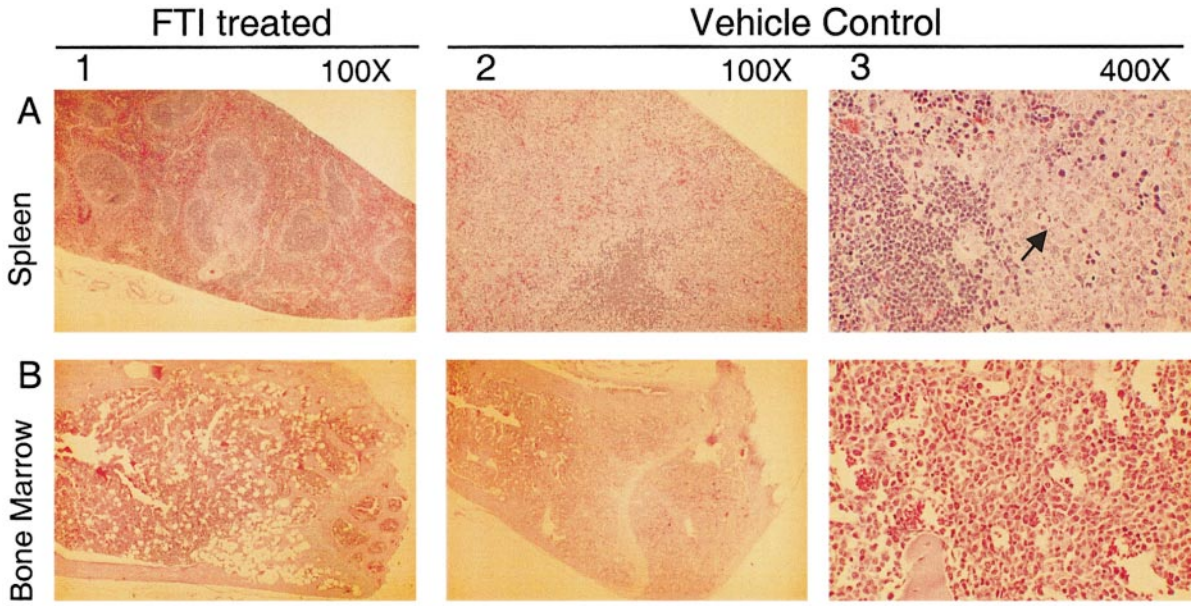

Figure 7. Histology of treated and diseased mice. Histopathologic analysis of bone marrow and spleen of mice injected with 10^6 BCR/ABL-BaF3 cells and treated with either SCH66336 (column 1) or vehicle alone (columns 2 and 3). Arrow represents site of infiltration with lymphoblasts. Magnification of photomicrographs is indicated.

nonhematopoietic tissues.[39] This model closely mimics human acute blast crisis of CML and, as such, provides a physiologically relevant test of a novel chemotherapeutic agent against a BCR/ABL-induced malignancy. To determine the relation of cell dose to time of disease onset, we injected groups of syngeneic Balb/c mice with graded doses of BCR/ABL-transformed BaF3 cells and observed the mice for signs of clinical deterioration. The time to morbidity decreased with an increase in number of BCR/ABL-BaF3 cells injected (Figure 6A). Given the reproducibility and consistency of disease induction by 10^6 cells, this dose was chosen for further experiments. Balb/c mice were injected with 10^6 BCR/ABL-BaF3 cells and treated by oral gavage, either with vehicle alone (control group) or 40 mg/kg SCH66336 delivered BID at approximately 12-hour intervals (Figure 6B). Mice treated with vehicle alone succumbed to leukemia and had disease latency equivalent to that of mice not subject to oral gavage, suggesting that the vehicle alone and the gavage treatment did not contribute significantly to morbidity. Disease was characterized by circulating blasts and marked splenomegaly. Average spleen size was 0.93 ± 0.06 g compared to FTI-treated mice in which the average spleen size was smaller than 0.1 g. Histopathologic analysis of untreated, diseased mice revealed infiltration of the spleen and bone marrow with hematopoietic blasts (Figure 7). The spleen demonstrated marked effacement of follicular architecture (Figure 7A-2). Bone marrow showed hypercellularity (Figure 7B-2). Higher magnification demonstrated infiltration of organs by blasts (Figure 7A-3, B-3). Blast infiltration was observed in nonhematopoietic tissues as well, including liver, small intestine, myocardium, and lung (not shown). All FTI-treated mice demonstrated modest weight loss (less than 10% initial body weight). Two mice became moribund soon after the cessation of treatment (days 33 and 40) and were killed. Although these mice survived longer than controls, they displayed typical signs of disease, including splenomegaly. However, all other treated mice survived and remained disease-free for more than 12 months (Figure 6B). Histopathologic analysis of organs from a surviving mouse demonstrates normal tissue architecture and no evidence of residual leukemia (Figure 7A-1, B-1). These results show that the FTI compound is highly effective at abrogating BCR/ABL-induced leukemia in vivo.

Inhibition of colony formation of human CML primary cells by SCH66336

Initial experiments with a less potent FTI compound in the same structural series as SCH66336 (SCH56582) proved to be effective at inhibiting colony formation of several human cell lines derived from patients with CML, including K562, KBM5, and KBM7 (data not shown). To address whether SCH66336 was effective against human BCR/ABL-transformed leukemia cells, primary cells from 2 patients with CML were exposed to SCH66336 and were assessed in methylcellulose colony assays. Colony formation was significantly inhibited by SCH66336, with an IC_{50} of between 500

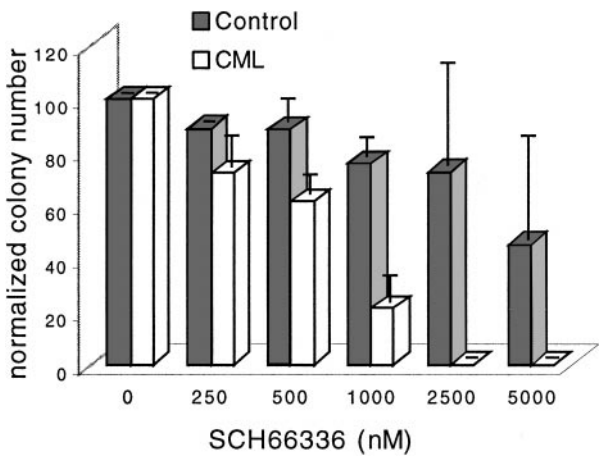

Figure 8. Methylcellulose colony assay of normal and CML human primary cells grown in SCH66336. Normal bone marrow (n = 3) and CML bone marrow or peripheral blood cells (n = 2) were grown in methylcellulose containing the indicated concentrations of SCH66336. Colony formation in the CML samples was significantly reduced by SCH66336, with an IC_{50} between 500 and 1000 nM. Normal samples were significantly less sensitive to SCH66336 colony inhibition. Colony counts were assessed on each individual sample at least twice, and results are presented as the average ± SD for colonies counted from duplicate plates under each condition.

and 1000 nM (Figure 8). In contrast, bone marrow cells from 3 healthy subjects were significantly less sensitive to colony inhibition by SCH66336 and showed only modest inhibition when the drug was used at concentrations 10 times higher than those that completely inhibited colonies from the patients with CML. These results provide evidence that the growth of human CML cells is inhibited by SCH66336 treatment at concentrations that do not significantly inhibit the growth of normal human cells, and they also provide evidence that FTIs have a therapeutic selectivity against BCR/ABL-transformed hematopoietic progenitors.

Discussion

In this study, we characterized the activity of SCH66336, a potent FTI initially developed for its activity against Ras farnesylation, which is in active clinical testing against solid tumors. We demonstrated the potent activity of this compound against BCR/ABL-BaF3 cells in vitro and against a murine model of BCR/ABL-induced acute leukemia. When tested against human primary cells, SCH66336 appears to spare hematopoietic colony formation from normal bone marrow at concentrations that completely suppress hematopoietic colonies from patients with CML, suggesting that this compound is an attractive candidate for testing in human clinical trials.

Although FTI compounds were designed to generate potent and specific blockade of Ras, it has become increasingly clear that other targets may be relevant to the chemotherapeutic efficacy of this class of pharmaceutical agents. FTIs antagonize the growth of Ras-dependent and Ras-independent tumor cells in a variety of cell culture and animal models.[44] Farnesylation of the H-Ras protein is blocked effectively by FTIs, but the K- and N-Ras proteins are subject to alternative prenylation by becoming substrates for the geranyl-geranyl protein transferase.[31] Despite alternative prenylation, FTI treatment blocks growth of K-ras and N-ras mutant tumors, and it is effective against cells transformed by Ras engineered to function independently of farnesylation (eg, by N-myristylation or geranyl-geranylation).[28,45] Additionally, tumors that harbor wild-type Ras can also be inhibited by FTI.[24] Thus the mechanism of action of this class of compounds appears more complicated than the simple inhibition of farnesylation and the alteration of subcellular localization of Ras. Immunoblotting demonstrates that K-Ras is the predominant form expressed in BaF3 cells. We were unable to demonstrate directly the blockade of K-Ras farnesylation by FTIs in BaF3 cells because of alternative prenylation (unpublished observation, 2000), but we demonstrated that farnesylation of another protein, DnaJ, was inhibited by FTI in these cells. Geranyl-geranylated Ras may have altered signaling properties. In BCR/ABL-BaF3 cells, FTI treatment reduced the levels of GTP-loaded Ras capable of interacting with the Raf-1 effector domain, suggesting that alternatively prenylated K-Ras might fail to associate with this effector molecule. We have demonstrated here that BCR/ABL activates DNA binding of the AP-1 transcription complex by a mechanism that can be inhibited by co-expression of a dominant-negative Ras or by treatment with FTI. These data are consistent with an interpretation that the FTIs suppress Ras signaling in BCR/ABL-transformed cells.

FTIs are more effective at inhibiting soft agar colony formation in BCR/ABL-BaF3 cells than is dominant-negative Ras (Figure 1), despite the comparable inhibition of Raf-1 association and AP-1 DNA binding. Therefore, we believe that inhibition of other critical farnesylated protein targets by FTIs must contribute significantly to the efficacy of these compounds against BCR/ABL-transformed cells. BCR/ABL activates Stat-5 DNA binding, which interestingly is also inhibited by FTI (unpublished observation, 2000); thus FTI may function by inhibiting multiple BCR/ABL effector pathways, either directly (ie, by Ras) or indirectly (ie, by Stat-5). Recently, Prendergast et al[29,46] demonstrated that inhibiting farnesylation of the RhoB protein leads to elevated levels of a geranyl-geranylated RhoB isoform with growth-suppressive properties. However, we have been unable to demonstrate significant inhibition of cell proliferation or enhanced sensitivity to apoptosis after ectopic expression of a geranyl-geranylated form of RhoB in BCR/ABL-transformed BaF3 cells (data not shown). BCR/ABL-BaF3 cells undergo a predominant G2/M blockade, which might be explained by the action of FTI against the CENP-E and CENP-F proteins—farnesylated mitotic kinesins that have recently been shown to be targets of FTIs.[27] The precise targets to account for the selective toxicity of FTIs against BCR/ABL-transformed cells remain to be defined.

In agreement with previous studies on the efficacy of SCH66336 in solid tumor models,[26] our results indicate that this FTI is therapeutically effective against BCR/ABL-induced leukemia in mice without causing significant toxicity. In this study, FTI treatment was administered daily and stopped approximately 1 week after the demise of the control group of animals. Although 2 of 15 treated mice succumbed to leukemia with a modestly delayed latency, all other mice have remained disease free for the duration of follow-up (more than 12 months). This result suggests that the FTI compound led to the eradication of leukemia cells in virtually all animals. FTIs are also active in a transgenic model of P190-BCR/ABL–induced acute leukemia. In this model, FTI treatment suppresses leukemic hematopoiesis, restores BCR/ABL-negative normal hematopoiesis, and prolongs survival for the duration of therapy.[47] The FTI compound SCH66336 is being tested in clinical trials of patients with a variety of solid tumors.[48] Our experiments demonstrating the suppression of hematopoietic colony formation in bone marrow samples from patients with CML, at doses that spare colony formation in samples taken from healthy subjects, suggest therapeutic selectivity for FTIs against BCR/ABL-transformed primary human cells. The results of the current study indicate that SCH66336 is a promising candidate for treating patients with BCR/ABL-derived leukemias and that it may be effective against myeloid leukemias that harbor activated Ras mutations.[49] Further preclinical animal studies will determine the merits of using FTI in combination with other treatments, such as specific tyrosine kinase inhibitors,[7] to combat BCR/ABL-induced leukemia.

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