In vitro and *In vivo* Activity of SKI-606, a Novel Src-Abl Inhibitor, against Imatinib-Resistant Bcr-Abl$^+$ Neoplastic Cells

Miriam Puttini,[1] Addolorata Maria Luce Coluccia,[1] Frank Boschelli,[2] Loredana Cleris,[3] Edoardo Marchesi,[3] Arianna Donella-Deana,[4] Shaheen Ahmed,[5] Sara Redaelli,[1] Rocco Piazza,[1] Vera Magistroni,[1] Federica Andreoni,[1] Leonardo Scapozza,[5] Franca Formelli,[3] and Carlo Gambacorti-Passerini[1,6]

[1]Department of Clinical Medicine, S. Gerardo Hospital-University of Milano-Bicocca, Monza, Italy; [2]Department of Oncology, Wyeth Research, Pearl River, New York; [3]Department of Experimental Oncology, National Cancer Institute, Milan, Italy; [4]Department of Biological Chemistry and Centro Interdipartimentale di Ricerca e Servizi per le Biotecnologie Innovative, National Research Centre, Institute of Neuroscience, University of Padova, Padova, Italy; [5]Pharmaceutical Biochemistry Group, School of Pharmaceutical Sciences, University of Geneva, University of Lausanne, Geneva, Switzerland; and [6]Department of Oncology, McGill University, Jewish General Hospital, Montreal, Quebec, Canada

Abstract

Resistance to imatinib represents an important scientific and clinical issue in chronic myelogenous leukemia. In the present study, the effects of the novel inhibitor SKI-606 on various models of resistance to imatinib were studied. SKI-606 proved to be an active inhibitor of Bcr-Abl in several chronic myelogenous leukemia cell lines and transfectants, with IC_{50} values in the low nanomolar range, 1 to 2 logs lower than those obtained with imatinib. Cells expressing activated forms of KIT or platelet-derived growth factor receptor (PDGFR), two additional targets of imatinib, were unaffected by SKI-606, whereas activity was found against PIM2. SKI-606 retained activity in cells where resistance to imatinib was caused by *BCR-ABL* gene amplification and in three of four Bcr-Abl point mutants tested. *In vivo* experiments confirmed SKI-606 activity in models where resistance was not caused by mutations as well as in cells carrying the Y253F, E255K, and D276G mutations. Modeling considerations attribute the superior activity of SKI-606 to its ability to bind a conformation of Bcr-Abl different from imatinib. (Cancer Res 2006; 66(23): 11314-22)

Introduction

The aberrantly activated tyrosine kinase Bcr-Abl, the product of the Philadelphia chromosome (Ph), is causally associated to the pathogenesis of chronic myelogenous leukemia (CML; ref. 1). This finding provided the rationale for the clinical success of the Bcr-Abl tyrosine kinase inhibitor imatinib mesylate (STI571, Gleevec/Glivec). Imatinib induces complete cytogenetic remissions (CCyR) with minimal toxicity in most patients with chronic-phase CML (2–4). However, patients in more advanced phases of CML and the majority of patients with Ph$^+$ acute lymphoblastic leukemia (ALL) either fail to respond to imatinib or quickly relapse following an initial response (5–7).

Resistance to imatinib (reviewed in ref. 8) is usually due to the overactivation of Bcr-Abl kinase activity that can be caused by the selection of cells harboring kinase point mutations of the *BCR-ABL* gene or presenting *BCR-ABL* gene amplification (9–11). Mutations or gene amplifications are not always observed in resistant cases. Instead, other mechanisms, such as up-regulation of drug efflux transporter (12, 13), down-regulation of influx pumps (14, 15), increased activity of downstream effectors, such as the Src tyrosine kinases (16, 17), and binding of imatinib by plasma or tissue proteins (18), have been postulated as additional mechanisms of resistance.

To overcome resistance to imatinib, different strategies were developed, including dose escalation of imatinib or combination with conventional cytotoxic drugs, but their success is limited, especially against patients harboring Bcr-Abl point mutations. To improve Bcr-Abl inhibition, several inhibitors with higher potency compared with imatinib have been identified, including dasatinib (BMS-354825) and nilotinib (AMN107), which show clinical activity in patients resistant to imatinib (19–21). Whereas nilotinib is derived from imatinib and binds to the same inactive conformation of Bcr-Abl (22), dasatinib is a dual Src-Abl inhibitor, which was crystallized in complex with the active conformation of Abl (23) and was also shown to dock in different conformations of the enzyme (24).

We describe here the *in vitro* and *in vivo* characterization of a new dual Src-Abl inhibitor, SKI-606 (25), against several CML models of resistance to imatinib.

Materials and Methods

Chemical compounds and reagents. SKI-606 is a novel substituted 4-anilino-3-quinolinecarbonitrile (Fig. 6*A*), with a molecular weight of 531 g/moL, and it was prepared as described (26). For *in vitro* experiments, a stock solution of SKI-606 was prepared at 1 and 10 mmol/L in DMSO (Sigma Chemical Co., St. Louis, MO), filtered, and stored at −20°C. Preparations used for animal experiments were made at the indicated doses in 0.2 mL vehicle containing 0.5% methylcellulose and 0.4% polysorbate 80 (Tween 80, Sigma Chemical), and the solutions were mixed continuously at 4°C. Imatinib mesylate was provided by Novartis Pharmaceuticals (AG, Basel, Switzerland) and prepared as described previously (2).

[γ-^{33}P]ATP was from Amersham Biosciences; R-angiotensin, RRRA-DDSDDDDD, RRKDLHDDEEDEAMSITA, and RRASVA were kindly provided by Dr. Oriano Marin (University of Padova, Padova, Italy).

Preparation of recombinant and native protein kinases. Human FLT-3 (amino acids 589-993, EntrezGene accession number NP_004110), ABL (amino acids 230-517, isoform a, NP_005148), and RET (amino acids 700-1,020, NP_066124) kinase domain cDNA sequences were cloned in pHisE baculovirus transfer vector (27), in *Bam*HI/*Sal*I, *Kpn*I/*Xba*I, and

Note: M. Puttini and A.M.L. Coluccia contributed equally to this work.

Requests for reprints: Carlo Gambacorti-Passerini, Internal Medicine, Department of Clinical Medicine and Prevention and Biotechnologies, University of Milano-Bicocca, via Cadore 48, 20052 Monza, Italy. Phone: 39-039-233-3539; Fax: 39-039-233-3539; E-mail: carlo.gambacorti@unimib.it.

doi:10.1158/0008-5472.CAN-06-1199

*Kpn*I/*Sal*I restriction sites, respectively. Anaplastic lymphoma kinase (ALK) sequence was cloned in pBlueBacHis2C vector (Invitrogen, Groningen, The Netherlands) as described (28). The sequence of all inserts was verified by DNA sequencing. Recombinant proteins were expressed in Sf9 cells with an NH_2-terminal hexahistidine tag to aid in purification, as described (27). Briefly, proteins from total lysate were fractionated in a DEAE-Sepharose column and positive fractions were identified by anti-HisG Western blotting, loaded on a nickel-chelate affinity column, and eluted with imidazole. The initial anion-exchange step is required to achieve homogeneity in the final preparation.

The nonreceptor tyrosine kinases Lyn, Fgr, Syk, and COOH-terminal Src kinase (Csk) were purified from rat spleen to near homogeneity as described previously (28). The serine-threonine protein kinases CK1 and CK2 were isolated from rat liver (29) and protein kinase A (PKA) was purchased from Sigma.

Radioactive kinase assay. Protein kinase assays were done in 30 μL of a medium containing 50 mmol/L Tris-HCl (pH 7.5), 5 mmol/L $MnCl_2$ (5 mmol/L $MgCl_2$ for c-Fgr, PKA, CK1, and CK2), 50 μmol/L [γ-^{33}P]ATP (specific activity, ~1,000 cpm/pmol), 10 units of each enzyme, and the following kinase-specific peptide substrates (200 μmol/L): R-angiotensin (tyrosine kinases), RRRADDSDDDDD (CK2), RRKDLHDDEEDEAMSITA (CK1), and RRASVA (PKA). The reactions were terminated after 10 minutes by spotting 25 μL of the incubation mixture onto P81 phosphocellulose paper, which was then processed as described elsewhere (30). One unit was defined as the amount of enzyme, which transferred 1 pmol phosphate/min to either 0.1 mg/mL polyGlu/Tyr (tyrosine kinases) or casein (protein kinases CK1 and CK2) or histone IIA, under standard conditions.

Inhibitor specificity profiling. Radioactive kinase assays were done at room temperature in the presence of substrate peptides, [γ-^{33}P]ATP, and 0.1 μmol/L SKI-606, using recombinant kinases obtained from various expression systems. Procedures for purification and assay of 30 of the 52 kinases have been described (31, 32). Kinase assays were done using ATP concentrations close to the K_m value for each kinase. All protein kinases were grouped accordingly into three categories (i.e., 5, 20, and 50 μmol/L ATP).

Cell lines and cell culture. The leukemic Bcr-Abl$^+$ cell lines (KCL22, K562, KU812, and Lama84) and the neoplastic Bcr-Abl$^-$ U937 cells were described previously (33). Ba/F3 cells expressing Tel-PDGFRβ kinase were kindly supplied by Dr. M. Carrol (University of Pennsylvania, Philadelphia, PA) and cultured as described previously (34). The human mast cell subline HMC-1^{560}, harboring a point mutation in the juxtamembrane domain of c-KIT causing an amino acid substitution G560V, was isolated as described previously (35) and kindly supplied by Dr. G. Nilsson (Uppsala University, Uppsala, Sweden) with the permission of Dr. J. Butterfield (Mayo Clinic Rochester, Rochester, MN). The human gastrointestinal stromal tumor cell line, GIST882, expressing an activating c-KIT mutation (exon 13, K642E) was obtained from Dr. J. Fletcher (Massachusetts Institute of Technology Cancer Center, Cambridge, MA; ref. 36). All cell lines were grown in RPMI 1640 (BioWhittaker Europe, Verviers, Belgium), supplemented with 10% fetal bovine serum (FBS; or 15% FBS for GIST882 cell line), 2 mmol/L L-glutamine, 100 units/mL penicillin G, 80 μg/mL gentamicin, and 20 mmol/L HEPES, in a humidified atmosphere at 37°C and 5% CO_2. The imatinib-resistant cell lines K562R and KCL22R were derived as described previously for Lama84R (11). Briefly, the imatinib-sensitive cell lines were cultured initially in the presence of 0.05 μmol/L imatinib; the dose was subsequently increased by 0.1 μmol/L every 3 to 4 weeks (1 week for KCL22). After few months of culture, a K562 subline growing at 0.6 μmol/L imatinib and a KCL22 subline growing at 3 μmol/L imatinib were isolated and designated K562R and KCL22R, respectively. The resistant cell lines were maintained in culture in medium supplemented with 0.6 μmol/L (Lama84R and K562R) or 3 μmol/L (KCL22R) imatinib. Murine pro-B cell line Ba/F3 was maintained in RPMI 1640 with 10% FBS, plus Chinese hamster ovary–conditioned supernatant as a source of interleukin-3 (IL-3).

Site-directed mutagenesis and generation of Ba/F3 transfectants. Ba/F3 p210 cells were obtained by transfecting the IL-3-dependent murine hematopoietic Ba/F3 cell line with a pcDNA3 vector containing p210 Bcr-Abl cDNA (37). The same plasmid was used as a template for the *in vitro* site-directed mutagenesis. Mutations D276G, Y253F, E255K, and T315I were generated with 'QuikChange XL Site-Directed Mutagenesis kit' (Stratagene, La Jolla, CA) following the standard protocol. For the stable transfection, 10^7 Ba/F3 cells were resuspended in 600 μL RPMI 1640, and 30 μg linearized plasmid in PBS were added. Cells were incubated on ice for 2 minutes and then electroporated (270 V, 960 μF) using a GenePulser XCell (Bio-Rad, Hercules, CA). Cells were kept on ice for 2 minutes and then transferred in 20 mL RPMI 1640 with FBS (10%) and IL-3 at standard concentrations (38). After 24 hours, 1 mg/mL G418 was added and the cells were cultured until day 14 when IL-3 was withdrawn. Cells were grown under selection with G418 for 4 weeks.

Proliferation assay. Serial dilutions of kinase inhibitors were prepared in cell culture medium in 96-well plates. Cells were then resuspended in complete medium and added to the plate at 10^4 per well. Cell proliferation was measured at 72 hours using the tritiated thymidine incorporation assay as described previously (2).

Immunoblotting. Ba/F3 p210 wild-type (WT) and mutants cells (2.5×10^6) were incubated in the presence of SKI-606 and imatinib at 0.01, 0.1, 1, and 10 μmol/L. After 2 hours of incubation, cells were washed 1× in PBS and then lysed in 250 μL lysis buffer [0.025 mol/L Tris (pH 8.0), 0.15 mol/L NaCl, 1% NP40 (w/v), 0.01 mol/L NaF, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L sodium orthovanadate (Na_3VO_4), 1 μg/mL leupeptin and aprotinin]. Total protein content of the supernatants was measured by Bradford assay. Equal amounts (30 μg) of total proteins were loaded on 7.5% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-phosphorylated tyrosine (4G10, Upstate Biotechnology, Lake Placid, NY) and anti-cAbl antibody (K-12, Santa Cruz Biotechnology, Santa Cruz, CA).

***In vivo* studies.** Five- to 7-week-old female CD1 *nu/nu* mice were purchased from Charles River Breeding Laboratories (Calco, Italy) kept under standard laboratory conditions according to the guidelines of the National Cancer Institute (NCI; Milan, Italy). This study was approved by the institutional ethics committee for laboratory animals used in experimental research of the NCI. Human KU812 Bcr-Abl$^+$ cells were suspended at 50 million cells in 0.5 mL PBS and this cell suspension was injected s.c. in the left flank of each animal. Murine Ba/F3 pro-B cells expressing Bcr-Abl WT or imatinib-resistant point mutants (Y253F, E255K, D276G, and T315I) were suspended at 10 million cells in 0.4 mL PBS and injected s.c. to syngeneic nude mice. Tumor weight and body weight were monitored twice or thrice weekly. Tumor weight was calculated by the formula tumor weight (mg) = ($d^2 \times D$ / 2), where d and D are the shortest and longest diameters of the tumor, respectively, measured in millimeters. Tumor weight was calculated considering tumor-bearing animals only. SKI-606 was given by oral gavage from day after cell infusion, at 8 days after cell injection when tumors entered growth phase or at 15 days to mice bearing measurable tumors as described in the figure legends. Placebo-treated animals received the same regimen with vehicle alone (0.5% methylcellulose-0.4% Tween 80).

Statistical analysis. Statistical analysis of tumor weights was done by Student's t test using GraphPad software analysis program (Prism, San Diego, CA). For analysis of tumor-free survival, data were compared by the log-rank test (39). Ps <0.05 were considered statistically significant and derived from two-sided statistical test.

Molecular modeling. SKI-606 was docked into the structure of c-Abl mouse (PDB entry: 1M52; ref. 40) in complex with PD173955 after removal of the inhibitor from the binding site. This specific conformation of Abl was chosen because of the fact that PD173955 is a dual Src-Abl inhibitor (41) as SKI-606 and that up to now neither a crystal structure of Abl complexed with a ligand scaffold more similar to SKI-606 nor an apo-structure is available. The docking was done using FlexX (42) with default variables. The binding site was defined by superimposing 1M52 onto 1IEP (c-abl mouse in complex with imatinib; ref. 40) taking into account all residues of 1M52 positioned 6.5 Å around imatinib of 1IEP. No further modifications to the active site were applied to accommodate SKI-606. The docking protocol was validated by reproducing the binding mode of PD173955 in the A-chain of the Abl crystal structure of 1M52 and the binding mode of imatinib in 1IEP. Thus, this docking protocol represents a fast and rather unbiased way to get

a binding mode hypothesis. A subsequent minimization of the docked ligand and the active site 6 Å around the docking position did not lead to any major conformational changes.

Results

SKI-606 is a potent inhibitor of Src family kinases. SKI-606, originally identified as a Src tyrosine kinase inhibitor, was subsequently found to also inhibit Abl tyrosine kinase (25). The ability of SKI-606 to prevent the kinase activation of selected tyrosine kinases was analyzed by testing *in vitro* the drug against various purified enzymes (Fig. 1*A*). SKI-606 was a picomolar inhibitor of two Src-related kinases (i.e., Fgr and Lyn with IC_{50}s of 0.174 and 0.850 nmol/L, respectively). It inhibits Abl kinase in a nanomolar range (IC_{50}, 2.4 nmol/L), as reported previously (25), and the Csk (IC_{50}, 314 nmol/L), which acts as endogenous inhibitor of Src family protein tyrosine kinases. The other tyrosine kinases (Syk, ALK, RET, and Flt-3) and serine-threonine kinases (PKA, CK1, and CK2) tested were inhibited only at micromolar concentrations (Fig. 1*A*). Data available from the literature on the inhibition of two of these kinases by dasatinib are also presented and show an activity similar to SKI-606 (43). To further enlarge the kinase specificity profile of SKI-606, a panel of 52 purified proteins belonging to serine-threonine or tyrosine kinase classes was incubated with 0.1 μmol/L inhibitor, a concentration that causes 95% of Abl kinase activity inhibition. As reported in Fig. 1*B*, c-Src- and Src-related Lck were fully inhibited. The serine-threonine kinase PIM2 was substantially (75%) but not completely inhibited. In addition, the serine-threonine kinase checkpoint kinase 2 was partially (52%) inhibited, whereas Csk kinase showed 58% of inhibition in this type of experiment. Thus, SKI-606 is a potent inhibitor of Src family and Abl tyrosine kinases, with lower activity against few other serine-threonine/tyrosine kinases in the panel of kinases examined.

SKI-606 is a nanomolar inhibitor of imatinib-resistant cell lines. SKI-606 was reported previously to reduce proliferation and survival of KU812 and K562 Bcr-Abl$^+$ CML cell lines (25). As indicated in Table 1, when the antiproliferative effect of SKI-606 and imatinib was compared in several human CML-derived cell lines, SKI-606 was more active than imatinib. In fact, SKI-606 inhibited these cell lines with IC_{50} values ranging from 1 to 20 nmol/L (Table 1) compared with 51 to 221 nmol/L for imatinib. These data agree with previously published results (33). Subsequently, we evaluated SKI-606 against two additional targets of imatinib, PDGFR and KIT. In PDGFR-transformed murine Ba/F3 cells (Table 1), a 50% inhibition was observed at 370 nmol/L SKI-606, 2 logs higher than imatinib (3 nmol/L). In addition, a mastocytosis HMC-1^{560} cell line and a gastrointestinal stromal tumor GIST882 cell line harboring activating mutations of c-KIT [a G560V mutation in the juxtamembrane domain (35) and a K642E in the exon 13 of c-KIT (36), respectively] were inhibited by SKI-606 only at micromolar concentrations, whereas imatinib IC_{50} values ranged between 19 and 29.5 nmol/L (Table 1). Data available from

A

Kinase	SKI-606 IC_{50} [nM]	dasatinib IC_{50} [nM] *
Fgr	0.174 ± 0.042	na
Lyn	0.850 ± 0.247	2.8
Abl	2.400 ± 0.700	0.6
Csk	314.2 ± 78.9	na
Syk	4228 ± 900	na
Alk	5720 ± 1115	na
Flt-3	>20000	na
Ret	>20000	na
PKA	>20000	na
CK1	>20000	na
CK2	>20000	na

B

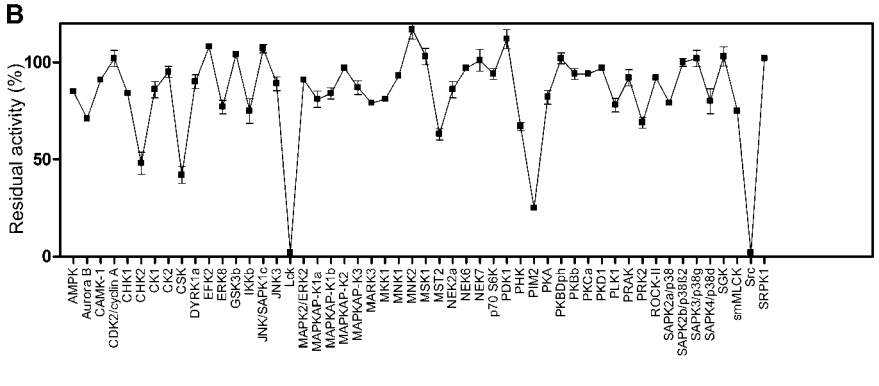

Figure 1. *A,* effect of SKI-606 and dasatinib on different protein kinases. IC_{50} values represent the mean of at least four separate determinations ± SD. Kinase activity was measured with a radioactive kinase assay using exogenous peptide substrates as detailed in Materials and Methods. Data on the same kinases available from literature are also reported. *, obtained from ref. 43. *na,* not available. *B,* residual activity (mean ± SD, percentage of control) of the listed protein kinases in presence of 0.1 μmol/L SKI-606. Kinase activity was measured with a radioactive kinase assay using exogenous peptide as described in Material and Methods.

Table 1. Comparison of SKI-606 and imatinib on cell proliferation

Kinase	SKI-606 (nmol/L)	Imatinib (nmol/L)
	IC_{50} ± SE (no. repeats)	IC_{50} ± SE (no. repeats)
WT-Ba/F3 + IL-3	570 ± 350 (4)	5,660 ± 2,760 (3)
Bcr-Abl (K562)	20 ± 2 (2)	221 ± 20 (2)
Bcr-Abl (Lama84)	1 ± 0.3 (6)	86 ± 8 (11)
Bcr-Abl (KU812)	3 ± 1 (2)	51 ± 5 (2)
Bcr-Abl (KCL22)	5 ± 1 (2)	70.5 (1)
Bcr-Abl (K562R, imatinib resistant)	28 ± 3 (2)	3,190 (1)
Bcr-Abl (Lama84R, imatinib resistant)	35 (1)	735 ± 380 (2)
Bcr-Abl (KCL22R, imatinib resistant)	150 (1)	2,170 ± 460 (2)
p210 Bcr-Abl (Ba/F3)	13 ± 4 (6)	401 ± 70 (11)
D276G Bcr-Abl (Ba/F3)	25 ± 15 (2)	1147 ± 231 (4)
Y253F Bcr-Abl (Ba/F3)	40 ± 22 (3)	1,888 ± 979 (4)
E255K Bcr-Abl (Ba/F3)	394 (1)	3,174 ± 1,211 (2)
T315I Bcr-Abl (Ba/F3)	1,800 ± 850 (2)	>10,000 (1)
Tel-PDGFRβ (Ba/F3)	370 ± 180 (5)	3.4 ± 0.9 (5)
c-KIT exon 13 mutant (GIST882)	6,000 (1)	29.5 (1)
c-KIT G560V ($HMC1^{560}$)	950 ± 450(2)	19 (1)
(U937)	3,500 ± 850 (5)	>10,000 (4)

NOTE: Dose-response curves were used to calculate IC_{50} values, expressed as mean ± SE. The antiproliferative activity was evaluated by [^{3}H]thymidine uptake.

the literature show that dasatinib too is active against KIT and PDGFR, with IC_{50} values of 7 and 50 nmol/L, respectively (44, 45). These data indicated that SKI-606 was inactive or only weakly active against cells transformed by PDGFR and KIT, in contrast to imatinib and dasatinib.

The efficacy of SKI-606 was also tested against imatinib-resistant cell lines, such as Lama84R, KCL22R, and K562R human cell lines (Table 1). The molecular mechanism of imatinib resistance of Lama84R cells is due to an amplification of Bcr-Abl fusion gene (11), whereas the underlying mechanism of KCL22R and K562R resistance has not been defined. SKI-606 was more active than imatinib in reducing the proliferation rate of K562R (IC_{50}, 28 versus 3,000 nmol/L), KCl22R (IC_{50}, 150 versus 2,000 nmol/L), and Lama84R (IC_{50}, 35 versus 700 nmol/L), respectively.

SKI-606 was also tested against murine pro-B Ba/F3 cells stably transformed by p210 Bcr-Abl WT or four imatinib-resistant point mutants (D276G, Y253F, E255K, and T315I; Fig. 2; Table 1). The compound inhibited the proliferation rate of WT, D276G, and Y253F transfectants in the low nanomolar range, having IC_{50} values ranging from 13 to 40 nmol/L (Y253F). The D276G and Y253F transfectants behaved similarly to the WT transfectant (Fig. 2*A*), in contrast to the results obtained with imatinib (Fig. 2*B*).

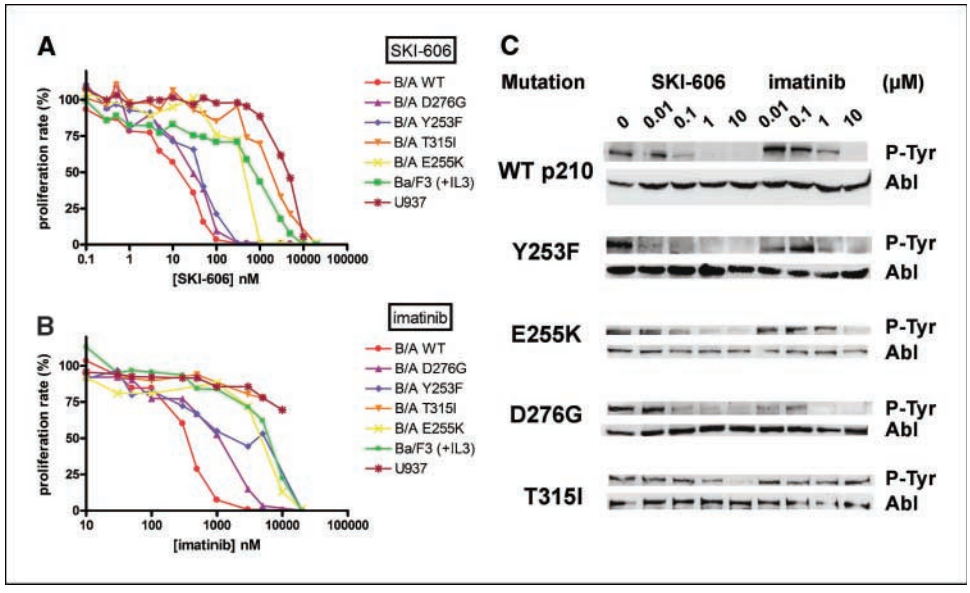

Figure 2. Effects of SKI-606 and imatinib on proliferation rate and Abl kinase activity of WT and mutant (D276G, Y253F, T315I, and E255K) Bcr-Abl–expressing Ba/F3 cells. *A* and *B*, Ba/F3 cells supplemented with IL-3, Ba/F3 cells expressing WT or mutant Bcr-Abl protein, and Bcr-Abl$^-$ U937 cells were plated in quintuplicates at 10^4 per well in 96-well plates with escalating concentrations of SKI-606 (0-20 μmol/L) or imatinib (0-20 μmol/L) for 3 days and evaluated using a [^{3}H]thymidine uptake assay. DMSO-treated controls were set as 100%. Error bars are omitted for clarity. *C, in vitro* inhibition of tyrosine phosphorylation of Bcr-Abl kinase. Whole-cell lysates were prepared from Ba/F3 cells after 2 hours of incubation with SKI-606 or imatinib and subjected to Western blot analysis with antibodies against phosphorylated tyrosine or Abl as described in Materials and Methods.

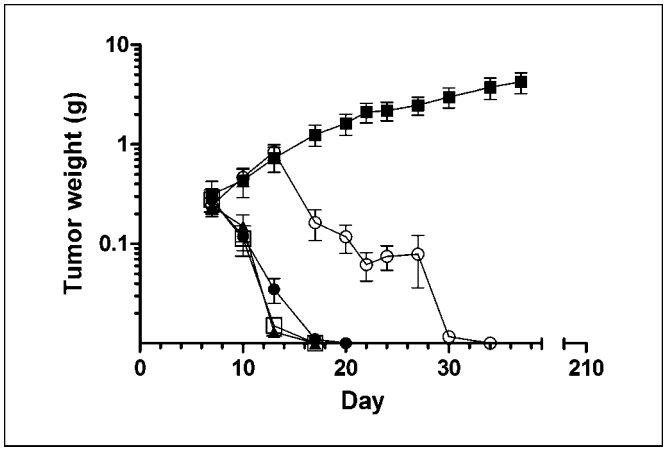

Figure 3. Effects of SKI-606 against KU812 leukemic cells injected in nude mice. Animals were treated 8 (▲,□, and ●) or 15 (○) days after tumor cell injection (mean tumor weight at day 8: 0.278 ± 0.203 mg; mean tumor weight at day 15: 0.840 ± 0.397 mg). Mice received SKI-606 at 75 mg/kg twice daily (▲ and ○) or 150 mg/kg once daily (□ and ●) or vehicle (■). SKI-606 was given continuously for 11 days, except in one group (●) where the treatment was given 5 days weekly. Tumors were monitored until day 210.

In E255K transfectants, the IC_{50} for SKI-606 was substantially higher (394 nmol/L) than in WT cells but lower than imatinib (3,174 nmol/L), whereas those observed for T315I transfectants were close to control cells. Whereas SKI-606 achieved 50% inhibition of the control Bcr-Abl$^-$ U937 cells at micromolar concentrations (3,500 nmol/L), the parental IL-3-dependent Ba/F3 cell line was inhibited with IC_{50} of 570 nmol/L, suggesting that SKI-606 could interfere with IL-3 signaling.

The inhibitory activity of SKI-606 and imatinib against WT Bcr-Abl and mutant kinases was also compared by immunoblotting analysis of total lysates derived from stable Ba/F3 transfectants, consistent with the antiproliferative activity of SKI-606 described above (Fig. 2*C*). Phosphorylation of Y253F was diminished at lower concentrations than observed for WT Bcr-Abl, whereas phosphorylation of the E255K and D276G mutants exhibited sensitivity to SKI-606 that was similar to WT Bcr-Abl. In contrast, whereas phosphorylation of the T315I mutant was inhibited by SKI-606, one to two order of magnitude higher concentration of SKI-606 was required to do so compared with WT Bcr-Abl.

SKI-606 is active against human KU812 xenografts in nude mice. It was shown previously that SKI-606 given at 75 mg/kg twice daily or 150 mg/kg once daily promoted complete regression of human K562 xenografts for up to 40 days (25). The *in vivo* efficacy of SKI-606 was tested in a KU812 human tumor xenograft model, in which we established previously the curative potential for imatinib (18). Mice bearing measurable tumors were treated with SKI-606 8 or 15 days after leukemic cell injection. In the latter condition (treatment after 15 days), imatinib is unable to eradicate the disease, whereas treatment at day 8 results in ~30% rate of relapse (18). In both models, SKI-606 led to a complete eradication of the tumors and all animals remained tumor-free for up to day 210 (Fig. 3).

Despite the therapeutic activity in this KU812 CML xenograft model, SKI-606 apparently produced a dose- and schedule-dependent weight loss. The dose of 150 mg/kg given continuously once daily for 11 days resulted in the highest toxicity, causing 25% to 30% of maximum body weight loss (BWL). The dose of 75 mg/kg twice daily had an intermediate toxicity, causing 15% to 20% of maximum BWL. The 5-day weekly regimen was better tolerated causing only a 15% of maximum BWL (data not shown).

Similar toxicity was not observed with the K562 model reported earlier (25), but these models differ from those earlier reported, and the mice used for the earlier study have a different genetic background. This likely represents a consequence of poorly understood host-tumor interactions in conjunction with aspects of inhibiting several Src family kinases in immunocompromised mice and are not good predictors of toxicity in humans.

SKI-606 is active against syngeneic Bcr-Abl WT and mutant Ba/F3 xenografts. In a syngeneic xenograft mouse model, pro-B murine Ba/F3 cells expressing p210 Bcr-Abl WT and three imatinib-resistant point mutants (Y253F, D276G, and T315I) were injected s.c. in nude mice. SKI-606 was given at 150 mg/kg once daily, 5 days weekly for a total of 11 days of treatment, starting

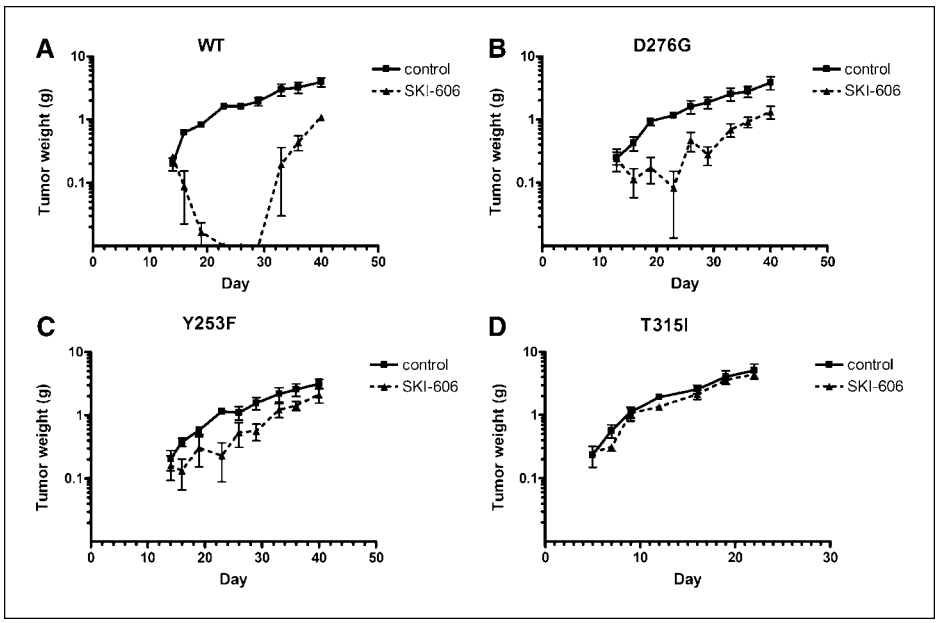

Figure 4. *In vivo* effects of SKI-606 against Ba/F3 cells harboring Bcr-Abl WT (*A*) or kinase point mutations D276G (*B*), Y253F (*C*), T315I (*D*) injected in nude mice. Groups of four animals were used. Treatment was started when tumors became measurable (mean tumor weight range, 131-375 mg). Tumor growth of untreated controls (*solid line*) or SKI-606-treated animals (*dashed line*). SKI-606 was given at 150 mg/kg once daily for 11 days (5 days weekly). *Points,* mean of the tumor growth in tumor-bearing animals; *bars,* SE.

when the tumors became measurable (tumor weight range, 131-375 mg). SKI-606-treated Bcr-Abl WT xenografts completely disappeared after the 1st week of treatment; however, two of four treated mice relapsed after a 14-day tumor-free window (Fig. 4*A*). The maximal effect of SKI-606 in D276G Ba/F3 xenografts was reached at the end of the 2nd week of treatment (day 23; Fig. 4*B*), when three of four mice had a complete tumor regression, but all subsequently relapsed. In Y253F xenografts (Fig. 4*C*), a statistically significant tumor growth inhibition of control versus SKI-606-treated animals was observed on day 23 (Ps = 0.0028) and day 29 (P = 0.039). SKI-606 treatment of mice bearing T315I xenografts did not inhibit tumor growth (Fig. 4*D*).

In other experiments, tumor-free survival and tumor growth were monitored in mice injected s.c. with Bcr-Abl$^+$ xenografts containing WT or mutant Bcr-Abl (E255K, Y253F, and D276G) and treated with SKI-606 1 day after tumor cell injection. The T315I mutant was excluded because of its high resistance to SKI-606 treatment in the previous experiments. Figure 5 shows that SKI-606 induced a statistically significant decrease in the rate of tumor growth and prolonged event-free survival of mice injected with Ba/F3 Bcr-Abl WT or with 253, 255, or 276 mutants, as described in the figure legend.

Modeling considerations on the activity of SKI-606. SKI-606 was found to dock inside the intermediate (1M52) conformation of Abl [Fig. 6*C*, for a comparison on how the docking solution of SKI-606 compared with respect to cocrystallized ligand of 1M52 (PD179355); see Fig. 6*B*], at difference with imatinib, which binds to the inactive conformation (1IEP) of Abl (40). Attempts to dock the compound in 1IEP led to a binding mode at the surface of the protein, which was not considered to be meaningful and was therefore discarded. The same observation was made trying to dock SKI-606 in an active conformation (2F4J) of Abl (46).

The activity of SKI-606 against the four Bcr-Abl point mutants analyzed can be rationalized as follows. The comparison of the docked solution of SKI-606 in 1M52 with the crystal structure of imatinib in 1IEP revealed that T315I is involved in direct electrostatic interactions with both SKI-606 (Fig. 6*D*) and imatinib. Therefore, a decrease in activity is expected for both molecules because of the steric hindrance caused by the bulky isoleucine as well as loss of the electrostatic interaction. Unlike T315, Y253 and E255, both located in the nucleotide binding loop, do not make any polar interaction with either SKI-606 or imatinib. However, the hydroxyl group of Y253 is involved in hydrogen bonding to N322, which stabilizes the conformation of the nucleotide binding loop in both inactive and intermediate conformations. The loss of the hydrogen bond probably leads to a bigger conformational change of the nucleotide binding loop in the inactive conformation of Abl

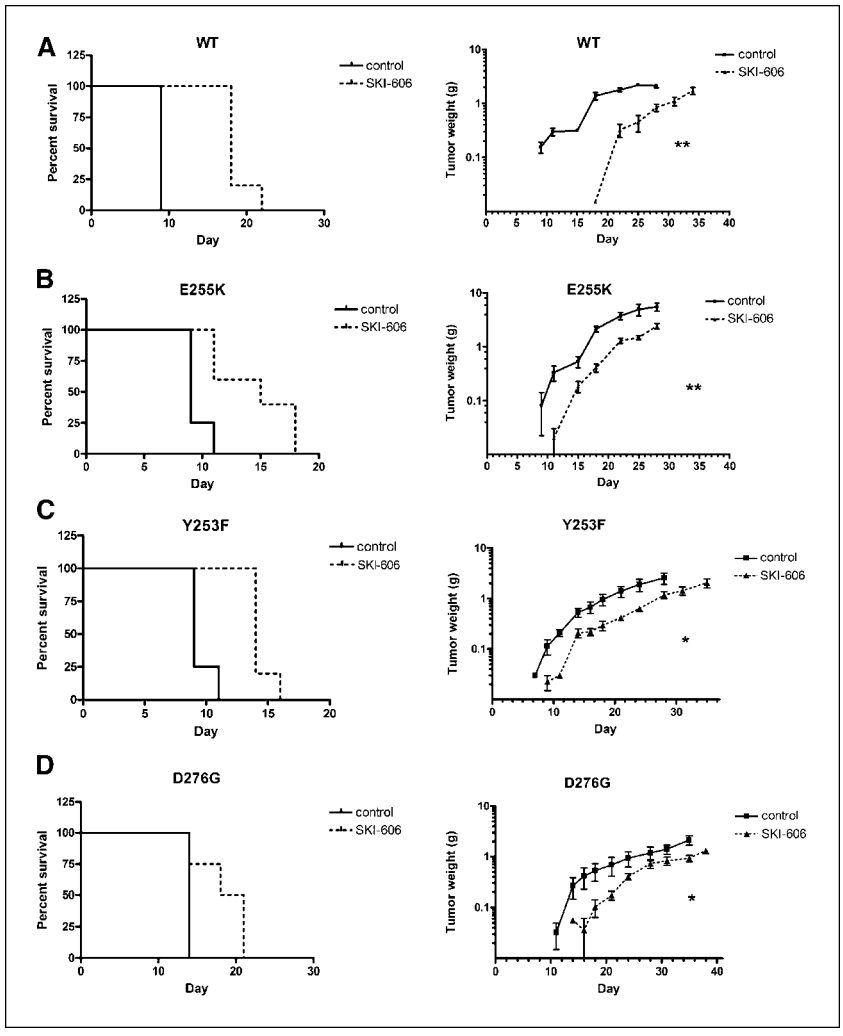

Figure 5. Effects of SKI-606 on tumor-free survival of nude mice injected with Ba/F3 cells harboring different Bcr-Abl mutations. Treatment was started the day after tumor cells injection on six animals per group. Results are presented as Kaplan-Meier plots of tumor-free survival (*left*) and tumor growth (*right*; *points*, mean; *bars*, SE) of Bcr-Abl WT (*A*), E255K (*B*), Y253F (*C*), D276G (*D*) xenografts given vehicle (*solid line*) or SKI-606 (*dashed line*). SKI-606 was given at 150 mg/kg once daily (*A* and *B*) or 75 mg/kg twice daily (*C* and *D*) from day 1 to 11. Statistical analysis done using log-rank test for survival curves (*left*) gives the following Ps: 0.0047 (*A*), 0.016 (*B*), 0.0027 (*C*), and 0.04 (*D*). The t test was used for tumor growth analysis. *, P = 0.011 to 0.05; **, P = 0.001 to 0.01.

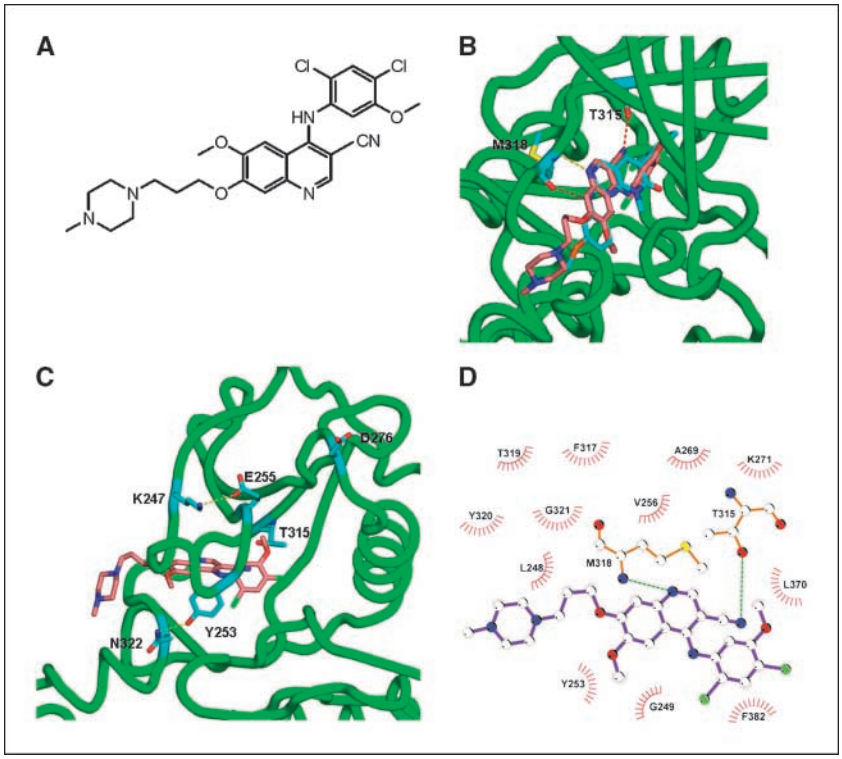

Figure 6. *A,* molecular structure of SKI-606. *B,* docked solution of SKI-606 (*pink carbons*) in 1M52 (*intermediate conformation of Abl*) superimposed onto the crystal structure of PD179355 (*blue carbons*). *Dotted yellow line,* common hydrogen bond formed with the protein (NH of M318); *dotted red lines,* hydrogen bonds unique to one of the ligands (O of M318 with NH of PD179355 and OH of T315 with N of CN of SKI-606). Residues T315 and M318 involved in hydrogen bonds are shown in color-coded sticks. *C,* 1M52, green tubes, with the docked solution of SKI-606 in color-coded sticks (*blue*, N; *red*, O; *pink*, C; *green*, Cl). Residues where mutations occur and their interaction partners within the protein are labeled and shown in color-coded sticks (*blue*, N; *red*, O; *blue*, C). *Dotted lines,* hydrogen bonds. *D,* schematic diagram of the interactions made by the docked solution of SKI-606 with the intermediate conformation of Abl. Protein residues forming hydrogen bonds are labeled and shown in ball and stick representation. Standard colors were used for heavy atoms. Hydrogen bonds (*dotted lines*); residues making van der Waals interactions with the inhibitor (*semicircled*).

compared with that of the intermediate conformation. This can be assumed because of the observation that in the inactive conformation the nucleotide binding loop is in contact with the activation loop and a conformational change of the nucleotide binding loop is likely to affect also the conformation of the activation loop. In the intermediate conformation of Abl, however, the activation loop is open and is not interacting with the nucleotide binding loop. E255 makes electrostatic interactions with K247, thereby stabilizing again the nucleotide binding loop. The loss of this interaction seems to have a more pronounced effect on the conformation of the nucleotide binding loop than for the Y253F mutation and is reflected by the higher IC_{50} values for both SKI-606 and imatinib (8).

D276 is close to the α-helix C and is involved in orientating the helix (37). The main effect of the D276G mutation is to destabilize the inactive conformation of Abl (37). A change in the orientation of the α-helix C is expected to affect only the binding of imatinib and not of SKI-606 because the predicted binding mode of SKI-606 suggests that it does not interact with this region.

The proposed binding mode of SKI-606 agrees with a recently published QSAR study (47) and in-house activity measurements[7] on SKI-606 derivatives. Docking studies on an Abl structure, which is very similar to 1M52 but which contains another ligand (2G2H; ref. 48), lead to a nearly identical binding mode to that observed while docking SKI-606 in 1M52.

Discussion

Imatinib treatment of CML patients dramatically changed their clinical course and the prognosis of this disease (3, 49, 50) in what is considered to be one of the major breakthroughs in modern medicine. Although most patients treated in chronic-phase now enjoy durable CCyR (50), most of them remain positive for the presence of Bcr-Abl$^+$ cells when tested by PCR (51). Under these conditions, the emergence of cells where further genetic alterations can cause resistance to imatinib represents a major potential threat. In addition, 10% to 15% of newly diagnosed patients fail to achieve CCyR and 1% to 2% of patients in CCyR lose it every year (50). Most Ph$^+$ ALL patients also fail to obtain durable responses with imatinib. For these reasons, the availability of drugs active in cases of resistance to imatinib is highly needed.

SKI-606 is, similarly to dasatinib, a dual Src-Abl inhibitor active against Bcr-Abl$^+$ CML cell lines (25). Our data confirm and expand the initial observation of Golas et al. (25) and investigate the activity of SKI-606 on several models of imatinib resistance. This molecule was tested in imatinib-resistant CML cell lines and Bcr-Abl transfectants. Because resistance to imatinib is not entirely explained by Bcr-Abl mutations (8, 52), we also analyzed three human CML lines with resistance to imatinib, which present no Bcr-Abl mutation. SKI-606 maintained activity in all three lines, including one (Lama84R), in which resistance is caused by *BCR-ABL* gene amplification, and two additional lines (K562R and KCL22R), in which no known mechanism have been identified. The KCL22R line proved to be the less sensitive one, but the IC_{50} value obtained (150 nmol/L) is within the concentration range that can be achieved *in vivo* (53).

We also selected four clinically relevant mutants of Bcr-Abl to be assayed with SKI-606. These four mutations recapitulate the different mechanisms by which mutations can cause resistance to imatinib: direct contact with the drug (T315I), P loop mutants (Y253F and E255K), and mutations that do not affect the contact with the drug, the P loop or the activation loop, but which cause resistance by shifting the equilibrium among the different

[7] Unpublished observation.

conformations of Bcr-Abl toward the active conformation (D276G; ref. 8). The latter class probably encompass the largest number of mutations identified thus far. Using these four mutants, SKI-606 activity was evident on D276G, Y253F, and partly in E255K and was absent in T315I mutants, thus confirming the T315I as the most difficult mutant to tackle. Modelling analysis also allowed us to explain this different sensitivity and to point to a further potential benefit of SKI-606 in comparison with imatinib: its ability to bind the conformational less restrictive intermediate conformation of the protein. To our knowledge, the study presented here is the first to show the different effects on SKI-606 binding of Abl mutations causing resistance to imatinib.

SKI-606 activity was also confirmed *in vivo* in two models. In the first one (s.c. KU812 model in nude mice), complete eradication of the tumor and cure of all animals could be obtained, even in a setting where imatinib was known to be unable to be curative (18). In the second model, Ba/F3 transfectants were injected in nude mice. In this model, some growth retardation was evident, although no leukemia eradication was achieved. The *in vivo* activity observed against the E255K mutants, despite the relatively high IC_{50} value observed *in vitro*, can be explained by the possibility that concentrations of SKI-606 sufficient to inhibit this mutation (0.39 μmol/L) can be reached *in vivo*, in contrast to imatinib, which has a higher IC_{50} against this mutant (>3 μmol/L). This model is particularly difficult because no animal could be cured even when using cells transformed with WT Bcr-Abl. Although the *in vivo* activity reported in the second model was limited against the Bcr-Abl mutations tested, the inherent limitations present in any model do not allow to conclude that the activity in patients with these mutations will be similarly limited.

It is known that imatinib achieves *in vivo* only a partial inhibition of Bcr-Abl enzymatic activity (4, 33); it is also known that Ph^+ stem cells represent the less sensitive subset of CML cells (54), probably because of a higher expression of the Bcr-Abl protein (55). It will be of interest to see if the availability of inhibitors more potent than imatinib, such as SKI-606, will allow a complete inhibition of Bcr-Abl to be obtained *in vivo* and will lead to greater number of patients to become PCR negative.

At difference with imatinib, no substantial inhibition of KIT and PDGFR was noted with SKI-606, thus placing this molecule in a class of its own (dual Src-Abl inhibitor devoid of activity on receptor tyrosine kinases). This different selectivity could result in fewer side effects than imatinib because many toxicities associated with imatinib (e.g., edema, muscle cramps, skin rash, pigmentation, endocrine abnormalities, and low-grade inhibition of normal hemopoiesis) can be tracked to the inhibition of PDGFR and/or KIT (56–59).

Dasatinib (BMS-354825) represents a recently registered dual Src-Abl inhibitor with a profile of activity similar to SKI-606. Dasatinib shows a slightly higher activity against Abl than SKI-606 (Fig. 1*A*). The lack of activity against receptors tyrosine kinases differentiates SKI-606 from dasatinib; this fact could render SKI-606 less toxic than dasatinib, including some recently described side effects (19, 21). In any case, a proper comparison of dasatinib and SKI-606 will require a direct clinical comparison of the two drugs as well as the availability of longer follow-up data on safety and efficacy.

Finally, the extended profile of activity done here allows us to conclude that SKI-606 is a fairly specific inhibitor, with a very limited number of targets outside the Abl and Src families of tyrosine kinase.

In conclusion, the data presented here show that SKI-606 exerts a specific activity against imatinib-resistant Bcr-Abl models. These data indicate that the potential activity of SKI-606 in the treatment of imatinib-resistant CML/Ph^+ ALL deserves the performance of dedicated phase I-II clinical trials.

Acknowledgments

Received 4/9/2006; revised 8/28/2006; accepted 9/12/2006.

Grant support: Associazione Italiana per la Ricerca sul Cancro, Min. San. Ricerca Finalizzata (2003); Centre National de la Recherche Scientifique and Minister of Education, University, and Research-COFIN and PRIN programs (2003 and 2004); European Union (Prokinase network #503467); Canadian Fund for Innovation (CFI); and NCI of Canada.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Diane Boschelli (Wyeth Research, Pearl River, NY) for kindly providing the SKI-606 compound; Protein Production and Assay Development team of the Division of Transduction Therapy at Dundee, coordinated by Hilary McLaughlan and James Hastie, for providing the protein kinases used in the specificity profile experiment; Dr. Jennifer Bain for doing the experiment; Drs. Rosalind Gunby and Luca Mologni (University of Milano-Bicocca, Monza, Italy) for kindly providing the ALK and RET recombinant proteins, respectively; Dr. Oriano Marin for the synthesis of the peptides; Drs. G. Nilsson and J. Butterfield for kindly providing the HMC-1^{560} human mastocytosis cell line; and Dr. J. Fletcher for providing the human GIST882 cell line.

References

1. Goldman JM, Melo JV. Chronic myeloid leukemia-advances in biology and new approaches to treatment. N Engl J Med 2003;349:1451–64.
2. le Coutre P, Mologni L, Cleris L, et al. *In vivo* eradication of human BCR/ABL-positive leukemia cells with an ABL kinase inhibitor. J Natl Cancer Inst 1999;91:163–8.
3. Kantarjian H, Sawyers C, Hochhaus A, et al. Hematologic and cytogenetic responses to imatinib mesylate in chronic myelogenous leukemia. N Engl J Med 2002;346:645–52.
4. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. N Engl J Med 2001;344:1031–7.
5. Talpaz M, Silver RT, Druker BJ, et al. Imatinib induces durable hematologic and cytogenetic responses in patients with accelerated phase chronic myeloid leukemia: results of a phase 2 study. Blood 2002;99:1928–37.
6. Sawyers CL, Hochhaus A, Feldman E, et al. Imatinib induces hematologic and cytogenetic responses in patients with chronic myelogenous leukemia in myeloid blast crisis: results of a phase II study. Blood 2002;99:3530–9.
7. Ottmann OG, Druker BJ, Sawyers CL, et al. A phase 2 study of imatinib in patients with relapsed or refractory Philadelphia chromosome-positive acute lymphoid leukemias. Blood 2002;100:1965–71.
8. Gambacorti-Passerini CB, Gunby RH, Piazza R, Galietta A, Rostagno R, Scapozza L. Molecular mechanisms of resistance to imatinib in Philadelphia-chromosome-positive leukaemias. Lancet Oncol 2003;4:75–85.
9. Gorre ME, Mohammed M, Ellwood K, et al. Clinical resistance to STI-571 cancer therapy caused by BCR-ABL gene mutation or amplification. Science 2001;293:876–80.
10. Hochhaus A, Kreil S, Corbin AS, et al. Molecular and chromosomal mechanisms of resistance to imatinib (STI571) therapy. Leukemia 2002;16:2190–6.
11. le Coutre P, Tassi E, Varella-Garcia M, et al. Induction of resistance to the Abelson inhibitor STI571 in human leukemic cells through gene amplification. Blood 2000;95:1758–66.
12. Mahon FX, Deininger MW, Schultheis B, et al. Selection and characterization of BCR-ABL positive cell lines with differential sensitivity to the tyrosine kinase inhibitor STI571: diverse mechanisms of resistance. Blood 2000;96:1070–9.
13. Illmer T, Schaich M, Platzbecker U, et al. P-glycoprotein-mediated drug efflux is a resistance mechanism of chronic myelogenous leukemia cells to treatment with imatinib mesylate. Leukemia 2004;18:401–8.
14. Thomas J, Wang L, Clark RE, Pirmohamed M. Active transport of imatinib into and out of cells: implications for drug resistance. Blood 2004;104:3739–45.
15. Crossman LC, Druker BJ, Deininger MW, Pirmohamed M, Wang L, Clark RE. hOCT 1 and resistance to imatinib. Blood 2005;106:1133–4; author reply 1134.
16. Donato NJ, Wu JY, Stapley J, et al. BCR-ABL independence and LYN kinase overexpression in chronic myelogenous leukemia cells selected for resistance to STI571. Blood 2003;101:690–8.

17. Ptasznik A, Nakata Y, Kalota A, Emerson SG, Gewirtz AM. Short interfering RNA (siRNA) targeting the Lyn kinase induces apoptosis in primary, and drug-resistant, BCR-ABL1(+) leukemia cells. Nat Med 2004;10:1187–9.
18. Gambacorti-Passerini C, Barni R, le Coutre P, et al. Role of α1 acid glycoprotein in the *in vivo* resistance of human BCR-ABL(+) leukemic cells to the abl inhibitor STI571. J Natl Cancer Inst 2000;92:1641–50.
19. Assouline S, Laneuville P, Gambacorti-Passerini C. Panniculitis during dasatinib therapy for imatinib-resistant chronic myelogenous leukemia. N Engl J Med 2006;354:2623–4.
20. Shah NP, Pasquini R, Hamerschlak N, et al. Dasatinib (D) vs high dose imatinib (IM) in patients (pts) with chronic phase chronic myeloid leukemia (CP-CML) resistant to imatinib. Results of CA180017 START-R randomized trial. J Clin Oncol: 2006 ASCO Annual Meeting Proceedings; 2006. Part I. Vol. 24. p. 6507.
21. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. N Engl J Med 2006;354:2531–41.
22. Weisberg E, Manley PW, Breitenstein W, et al. Characterization of AMN107, a selective inhibitor of native and mutant Bcr-Abl. Cancer Cell 2005;7:129–41.
23. Tokarski JS, Newitt JA, Chang CY, et al. The structure of dasatinib (BMS-354825) bound to activated ABL kinase domain elucidates its inhibitory activity against imatinib-resistant ABL mutants. Cancer Res 2006;66:5790–7.
24. Gambacorti-Passerini C, Gasser M, Ahmed S, Assouline S, Scapozza L. Abl inhibitor BMS354825 binding mode in Abelson kinase revealed by molecular docking studies. Leukemia 2005;19:1267–9.
25. Golas JM, Arndt K, Etienne C, et al. SKI-606, a 4-anilino-3-quinolinecarbonitrile dual inhibitor of Src and Abl kinases, is a potent antiproliferative agent against chronic myelogenous leukemia cells in culture and causes regression of K562 xenografts in nude mice. Cancer Res 2003;63:375–81.
26. Boschelli DH, Ye F, Wang YD, et al. Optimization of 4-phenylamino-3-quinolinecarbonitriles as potent inhibitors of Src kinase activity. J Med Chem 2001;44:3965–77.
27. Mologni L, Sala E, Riva B, et al. Expression, purification, and inhibition of human RET tyrosine kinase. Protein Expr Purif 2005;41:177–85.
28. Donella-Deana A, Marin O, Cesaro L, et al. Unique substrate specificity of anaplastic lymphoma kinase (ALK): development of phosphoacceptor peptides for the assay of ALK activity. Biochemistry 2005;44:8533–42.
29. Meggio F, Deana AD, Pinna LA. Endogenous phosphate acceptor proteins for rat liver cytosolic casein kinases. J Biol Chem 1981;256:11958–61.
30. Glass DB, Masaracchia RA, Feramisco JR, Kemp BE. Isolation of phosphorylated peptides and proteins on ion exchange papers. Anal Biochem 1978;87:566–75.
31. Davies SP, Reddy H, Caivano M, Cohen P. Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000;351:95–105.
32. Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. Biochem J 2003;371:199–204.
33. Gambacorti-Passerini C, le Coutre P, Mologni L, et al. Inhibition of the ABL kinase activity blocks the proliferation of BCR/ABL$^+$ leukemic cells and induces apoptosis. Blood Cells Mol Dis 1997;23:380–94.
34. Gunby RH, Cazzaniga G, Tassi E, et al. Sensitivity to imatinib but low frequency of the TEL/PDGFRβ fusion protein in chronic myelomonocytic leukemia. Haematologica 2003;88:408–15.
35. Sundstrom M, Vliagoftis H, Karlberg P, et al. Functional and phenotypic studies of two variants of a human mast cell line with a distinct set of mutations in the c-kit proto-oncogene. Immunology 2003;108:89–97.
36. Tuveson DA, Willis NA, Jacks T, et al. STI571 inactivation of the gastrointestinal stromal tumor c-KIT oncoprotein: biological and clinical implications. Oncogene 2001;20:5054–8.
37. Piazza RG, Magistroni V, Gasser M, et al. Evidence for D276G and L364I Bcr-Abl mutations in Ph$^+$ leukaemic cells obtained from patients resistant to imatinib. Leukemia 2005;19:132–4.
38. Bazzoni G, Carlesso N, Griffin JD, Hemler ME. Bcr/Abl expression stimulates integrin function in hematopoietic cell lines. J Clin Invest 1996;98:521–8.
39. Peto R, Pike MC, Armitage P, et al. Design and analysis of randomized clinical trials requiring prolonged observation of each patient. I. Introduction and design. Br J Cancer 1976;34:585–612.
40. Nagar B, Bornmann WG, Pellicena P, et al. Crystal structures of the kinase domain of c-Abl in complex with the small molecule inhibitors PD173955 and imatinib (STI-571). Cancer Res 2002;62:4236–43.
41. Wisniewski D, Lambek CL, Liu C, et al. Characterization of potent inhibitors of the Bcr-Abl and the c-kit receptor tyrosine kinases. Cancer Res 2002;62:4244–55.
42. Rarey M, Kramer B, Lengauer T, Klebe G. A fast flexible docking method using an incremental construction algorithm. J Mol Biol 1996;261:470–89.
43. O'Hare T, Pollock R, Stoffregen EP, et al. Inhibition of wild-type and mutant Bcr-Abl by AP23464, a potent ATP-based oncogenic protein kinase inhibitor: implications for CML. Blood 2004;104:2532–9.
44. Chen Z, Lee FY, Bhalla KN, Wu J. Potent inhibition of platelet-derived growth factor-induced responses in vascular smooth muscle cells by BMS-354825 (dasatinib). Mol Pharmacol 2006;69:1527–33.
45. Schittenhelm MM, Shiraga S, Schroeder A, et al. Dasatinib (BMS-354825), a dual SRC/ABL kinase inhibitor, inhibits the kinase activity of wild-type, juxtamembrane, and activation loop mutant KIT isoforms associated with human malignancies. Cancer Res 2006;66:473–81.
46. Young MA, Shah NP, Chao LH, et al. Structure of the kinase domain of an imatinib-resistant Abl mutant in complex with the Aurora kinase inhibitor VX-680. Cancer Res 2006;66:1007–14.
47. Thaimattam R, Daga PR, Banerjee R, Iqbal J. 3D-QSAR studies on c-Src kinase inhibitors and docking analyses of a potent dual kinase inhibitor of c-Src and c-Abl kinases. Bioorg Med Chem 2005;13:4704–12.
48. Levinson NM, Kuchment O, Shen K, et al. A Src-like inactive conformation in the abl tyrosine kinase domain. PLoS Biol 2006;4:e144.
49. O'Brien SG, Guilhot F, Larson RA, et al. Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia. N Engl J Med 2003;348:994–1004.
50. Gambacorti C, Talpaz M, Sawyers CL, et al. Five year follow-up results of a phase II trial in patients with late chronic phase (L-CP) chronic myeloid leukemia (CML) treated with imatinib who are refractory/intolerant of interferon-α [abstract 1089]. ASH Annual Meeting Abstracts 2005;106:1089.
51. Hughes TP, Kaeda J, Branford S, et al. Frequency of major molecular responses to imatinib or interferon alfa plus cytarabine in newly diagnosed chronic myeloid leukemia. N Engl J Med 2003;349:1423–32.
52. Cortes J. Overcoming drug resistance in chronic myeloid leukemia. Curr Opin Hematol 2006;13:79–86.
53. Golas JM, Lucas J, Etienne C, et al. SKI-606, a Src/Abl inhibitor with *in vivo* activity in colon tumor xenograft models. Cancer Res 2005;65:5358–64.
54. Elrick LJ, Jorgensen HG, Mountford JC, Holyoake TL. Punish the parent not the progeny. Blood 2005;105:1862–6.
55. Jiang X, Saw KM, Eaves A, Eaves C. Leukemic stem cells of chronic phase CML patients possess uniquely elevated BCR-ABL kinase activity and acquire spontaneous BCR-ABL kinase domain mutations at a high frequency [abstract 438]. ASH Annual Meeting Abstracts 2005;106:438.
56. Gambacorti-Passerini C, Tornaghi L, Cavagnini F, et al. Gynaecomastia in men with chronic myeloid leukaemia after imatinib. Lancet 2003;361:1954–6.
57. Tsao AS, Kantarjian H, Cortes J, O'Brien S, Talpaz M. Imatinib mesylate causes hypopigmentation in the skin. Cancer 2003;98:2483–7.
58. Leveen P, Pekny M, Gebre-Medhin S, Swolin B, Larsson E, Betsholtz C. Mice deficient for PDGF B show renal, cardiovascular, and hematological abnormalities. Genes Dev 1994;8:1875–87.
59. Rajaraman S, Davis WS, Mahakali-Zama A, Evans HK, Russell LB, Bedell MA. An allelic series of mutations in the Kit ligand gene of mice. II. Effects of ethylnitrosourea-induced Kitl point mutations on survival and peripheral blood cells of Kitl(Steel) mice. Genetics 2002;162:341–53.