Pharmacologic Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase/Mitogen-activated Protein Kinase Inhibitors Interact Synergistically with STI571 to Induce Apoptosis in Bcr/Abl-expressing Human Leukemia Cells

Chunrong Yu, Geoffrey Krystal, Lyuba Varticovksi, Robert McKinstry, Mohamed Rahmani, Paul Dent, and Steven Grant

Division of Hematology/Oncology [C. Y., G. K., M. R., S. G.], and the Department of Radiation Oncology [P. D., R. M.], Medical College of Virginia, Richmond, Virginia 23298, and the Department of Medicine, Tufts University, Boston, Massachusetts 02135 [L. V.]

ABSTRACT

Interactions between the kinase inhibitor STI571 and pharmacological antagonists of the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK)/mitogen-activated protein kinase (MAPK) cascade have been examined in human myeloid leukemia cells (K562 and LAMA 84) that express the Bcr-Abl kinase. Exposure of K562 cells to concentrations of STI571 that minimally induced apoptosis (e.g., \( \sim 200 \) nM) resulted in early suppression (i.e., at 6 h) of p42/44 MAPK phosphorylation followed at later intervals (i.e., \( \geq 24 \) h) by a marked increase in p42/44 MAPK phosphorylation/activation. Coadministration of a nontoxic concentration of the MEK1/2 inhibitor PD184352 (5 \( \mu \)M) prevented STI571-mediated activation of p42/44 MAPK. Cells exposed to STI571 in combination with PD184352 for 48 h demonstrated a very dramatic increase in mitochondrial dysfunction (e.g., loss of \( \Delta \Psi \)m and cytosolic cytochrome c release) associated with procaspase-3 activation, poly(ADP-ribose) polymerase cleavage, and the appearance of the characteristic morphological features of apoptosis. Similar results were obtained using other pharmacological MEK1/2 inhibitors (e.g., PD 98059 and U0126) as well as another leukemic cell line that expresses Bcr-Abl (e.g., LAMA 84). However, synergistic induction of apoptosis by STI571 and PD184352 was not observed in human myeloid leukemia cells that do not express the Bcr-Abl kinase (e.g., HL-60 and U937) nor in normal human peripheral blood mononuclear cells. Synergistic potentiation of STI571-mediated lethality by PD184352 was associated with multiple perturbations in signaling and apoptotic regulatory pathways, including caspase-dependent down-regulation of Bcr-Abl and Bcl-2; caspase-independent down-regulation of Bcl-xL and Mcl-1; activation of JNK, p38 MAPK, and p44(ERK)2; and diminished phosphorylation of Stat5 and CREB. Significantly, coexposure to PD184352 strikingly increased the lethality of a pharmacologically achievable concentration of STI571 (i.e., \( 1–2 \mu \)M) in resistant K562 cells expressing marked increases in Bcr-Abl protein levels. Together, these findings raise the possibility that treatment of Bcr-Abl-expressing cells with STI571 elicits a cytotoxic MAPK activation response and that interruption of the latter pathway (e.g., by pharmacological MEK1/2 inhibitors) is associated with a highly synergistic induction of mitochondrial damage and apoptosis. They also indicate that in the case of Bcr-Abl-positive cells, simultaneous interruption of two signal transduction pathways may represent an effective antileukemic strategy.

INTRODUCTION

The Bcr/Abl kinase is an oncogenic fusion protein that arises as a consequence of the joining of varying NH2-terminal sequences of the Bcr\(^2\) gene on chromosome 22 with COOH-terminal sequences (exons 2–11) of the abl gene on chromosome 9 (1). This fusion protein occurs in 95% of CML and 10–15% of acute lymphoblastic leukemia patients (2). As a result of alterations in both inter- and intramolecular interactions, the protein tyrosine kinase domain of the Bcr/Abl fusion protein becomes constitutively activated, an event that is required for malignant transformation (3). There is compelling evidence implicating constitutive activation of the Bcr/Abl oncogene in the pathogenesis of these disorders, including the observations that expression of the p210 Bcr/Abl fusion protein in murine hematopoietic cells results in a disease resembling CML in host mice (4–6). Among other actions, the Bcr/Abl kinase protects hematopoietic progenitor cells from spontaneous apoptosis as well as that induced by various noxious stimuli, including chemotherapeutic drugs (7). Although the precise mechanism(s) by which Bcr/Abl exerts its antiapoptotic properties is (are) unknown, several candidate downstream mediators have been proposed, including increased expression of the antiapoptotic proteins Bcl-xL (8) and induction of nuclear factor \( \kappa B \) (9).

Given the well-defined role of the Bcr/Abl kinase in CML and related disorders, it represents a very attractive molecular target for pharmacological intervention. Recently, considerable attention has focused on CGP57148B, currently referred to as STI571, an inhibitor of the Bcr/Abl, Kit, and platelet-derived growth factor receptor kinases (10). In \( \textit{in vitro} \) studies, STI571 has been shown to inhibit the growth of Bcr/Abl-positive leukemic cells at micromolar concentrations (11). Interestingly, exposure of such cells to STI571 promotes leukemic cell apoptosis (12), suggesting that Bcr/Abl not only confers resistance to drugs (7) but might also sensitize these cells to other cytotoxic therapies (13). In addition, preclinical studies have demonstrated that the combination of STI571 with established chemotherapeutic drugs (e.g., ara-C) results in enhanced toxicity in Bcr/Abl-positive leukemias (14, 15). These findings raise the possibility that combining STI571 with such agents might lead to enhanced activity in CML and/or circumvention of drug resistance. In this context, Vigneri and Wang (16) reported recently that coadministration of STI571 with leptomycin, an inhibitor of the nuclear export sequence receptor, resulted in increased killing of cells expressing Bcr/Abl. However, in this study optimal killing occurred in cells exposed to 10 \( \mu \)M STI571, which is above

The abbreviations used are: BCR, breakpoint cluster region; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase; PARP, poly(ADP-ribose) polymerase; Abl, Abelson murine leukemia; CML, chronic myelogenous leukemia; JNK, c-Jun-NH2-terminal kinase; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling; DiOC6, 3,3-dihexyloxacarbocyanine iodide; PBS-T, PBS-Tween; EMSA, electrophoretic mobility shift assay; MMP, mitochondrial membrane potential.
concentrations obtained in the plasma of patients receiving this agent (13).

In addition to tyrosine kinases such as Bcr/Abl, apoptosis and survival are also regulated by the activity of multiple other signal transduction pathways, particularly the MAPK cascade. The MAPK cascade consists of a superfamily of three parallel signal transduction modules converging on the serine/threonine kinases JNK, p24/44 MAPK (ERK), and p38 MAPK (17). These kinases are activated by a variety of stimuli and are intimately involved in diverse cellular processes including responses to DNA damage or osmotic shock, mitogenic stimuli, cell differentiation and survival, among others (18). Although exceptions occur, activation of JNK and p38 MAPK are generally associated with induction of apoptosis, whereas p24/44 MAPK exerts cytoprotective effects (19). Efforts to delineate the role of p42/44 MAPK in various cellular functions has been facilitated by the development of several pharmacological inhibitors of the enzymes that activate p42/44 (i.e., the MAPK kinases MEK1/2), including PD98059 (20), U0126 (21), and PD184352. The latter agent has attracted attention in view of its ability to inhibit p42/44 MAPK activity and to block human colon carcinoma growth in an in vivo model (22). The observation that MEK1/2 inhibitors potentiate the antitumor activity of various cytotoxic agents, including ara-C (23), cisplatin (24), and paclitaxel (25, 26), suggests a possible role for MEK1/2 inhibitors in the treatment of human malignancies.

The contribution of the ERK/MAPK cascade in the antiapoptotic actions of Bcr/Abl remains to be fully elucidated. For example, it has been shown that in fibroblasts and hematopoietic cells, JNK represents a primary target in Bcr/Abl-mediated transformation, although the Ras/MEK/MAPK pathway may be involved in this process (27). Other studies have demonstrated that MAPK is phosphorylated in hematopoietic cells constitutively expressing Bcr/Abl (28). Moreover, interference with MEK/MAPK activation has been implicated in apoptosis induction in Bcr/Abl-positive cells, including that occurring in response to STI571 (12, 29). Collectively, such findings raise the possibility that disruption of MEK/MAPK signaling might enhance the lethality of STI571 toward CML progenitors. To address this issue, we have examined interactions between pharmacological MEK1/2 inhibitors and STI571 in Bcr/Abl-positive human leukemia cells. Our results indicate that contrary to expectations, treatment of these cells with STI571 results in a delayed increase in p42/44 MAPK activation. Furthermore, coadministration of a marginally toxic concentration of STI571 with several MEK1/2 inhibitors is associated with a very marked increase in mitochondrial dysfunction and caspase activation, as well as a highly synergistic potentiation of apoptosis. Significantly, similar interactions occur in STI571-resistant cells that express high levels of the Bcr/Abl protein. Together, these findings suggest that the MEK/MAPK cascade exerts a cytoprotective effect in Bcr/Abl-positive leukemia cells exposed to STI571, and raise the possibility that combining this agent with pharmacological MEK1/2 inhibitors may represent a novel therapeutic strategy in CML and related disorders.

MATERIALS AND METHODS

Cells. K562, HL60, and U937 human leukemia cells were purchased from American Type Culture Collection, Rockville, MD. LAMA 84 cells were purchased from the German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). All were cultured in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, l-glutamate, penicillin, streptomycin, and 10% heat-inactivated FCS (Hyclone, Logan, UT). They were maintained in a 37°C, 5% CO2, fully humidified incubator, passed twice weekly, and prepared for experimental procedures when in log-phase growth (cell density ≤ 4 × 10^6 cells/ml).

Multidrug-resistant K562R cells were derived from the parental line by subculturing in progressively higher concentrations of doxorubicin as described previously (30). They were cultured in the absence of doxorubicin before all of the experimental procedures. In addition, STI571-resistant K562 cells, designated K562-R-STI, were generated by subculturing K562 cells in progressively higher concentrations of STI571. These cells are maintained under selection pressure in medium containing 1 μM of STI571. For studies involving the K562-R-STI line, cells are washed free of drug and resuspended in drug-free medium 48 h before experimentation.

Reagents. STI571 was kindly provided by Dr. Elizabeth Buchdunger, Novartis Pharmaceuticals, Basel, Switzerland, and prepared as a 10 μM stock solution in sterile DMSO (Sigma Chemical Co., St. Louis, MO). Verapamil was purchased from Sigma Chemical Co., stored in light-protected containers at -20°C, and dissolved in sterile 100% ethanol as a 0.1 μM stock solution before use. LAMA 84, K562, K562-R-STI, K562R, and Bcr/Abl positive H11003 cells were purchased from the German Collection of Microorganisms and Cell Cultures, and were cultured in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% heat-inactivated FCS (Hyclone, Logan, UT). They were washed twice in PBS, resuspended in 50 μl of lysis buffer containing 75 mM NaCl, 8 mM Na2HPO4, 1 mM NaH2PO4, 1 mM EDTA, and 350 μg/ml digitonin. The lysates were centrifuged at 12,000 × g for 5 min, and the supernatant was collected and added to an equal volume of 2 X LAEMMlI buffer. The protein samples were quantified and separated by 15% SDS-PAGE.

Preparation of S-100 Fractions and Assessment of Cytochrome C Release. K562 cells were harvested after drug treatment as described previously (31) by centrifugation at 600 × g for 10 min at 4°C and washed in PBS. Cells (4 × 10^6) were lysed by incubating for 3 min in 100 μl of lysis buffer containing 75 mM NaCl, 8 mM Na2HPO4, 1 mM NaH2PO4, 1 mM EDTA, and 350 μg/ml digitonin. The lysates were centrifuged at 12,000 × g for 5 min, and the supernatant was collected and added to an equal volume of 2 X LAEMMlI buffer. The protein samples were quantified and separated by 15% SDS-PAGE. Western Analysis. A minor modification of a method described previously was used (32). After treatment, whole cell pellets (1 × 10^7 cells/condition) were washed twice in PBS, resuspended in 30 μl of PBS, lysed by the addition of 50 μl 2 X Laemmli buffer [1X = 30 mM Tris-base (pH 6.8), 2% SDS, 2.88 mM  β-mercaptoethanol, and 10% glycerol], and briefly sonicated. Homogenates were quantified using Coomasie protein assay reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μg) were boiled for 10 min, separated by SDS-PAGE (5% stacker and 10% resolving), and electroblotted to nitro-
cellulose. The blots were stained in 0.1% amido black and destained in 5% acetic acid to ensure transfer and equal loading. After blocking in PBS-T (0.05%) and 5% milk for 1 h at 22°C, the blots were incubated in fresh blocking solution with an appropriate dilution of primary antibody for 4 h at 22°C. The source and dilution of antibodies were as follows: Bel-2 1:200, mouse monoclonal, Dako, Carpinteria, CA; Bcl-xl 1:1000, rabbit polyclonal, Santa Cruz Biotechnology; XIAP 1:1000, rabbit polyclonal, R & D Systems, Minneapolis, MN; Mcl-1 1:1000, mouse monoclonal, PharMingen, San Diego, CA; ERK 1/2 1:1000, rabbit polyclonal, Cell Signaling Technology, Beverly, MA; phospho-ERK 1/2 (thr202/tyr204) 1:1000, rabbit polyclonal, Cell Signaling Technology; JNK 1:1000, rabbit polyclonal, Santa Cruz Biotechnology; phospho-JNK 1:1000, mouse monoclonal, Santa Cruz Biotechnology; phospho-p38 MAPK 1:1000, rabbit polyclonal, Cell Signaling Technology; phospho-cdk2 1:1000, rabbit polyclonal, Cell Signaling Technology; phospho-CREB 1:1000, rabbit polyclonal, Upstate Biotechnology, Lake Placid, NY; phospho-tyrosine (PY20) 1:300, mouse monoclonal, Transduction Laboratories; cleaved caspase 3 and phospho-Stat5, 1:1000, rabbit polyclonal, Santa Cruz Biotechnology and labeled with [32P]ATP (5,000 Ci/mmol, Amersham Pharmacia Biotech) using T4 polynucleotide kinase (Promega) and 32P-labeled oligonucleotide probe dissolved in 15 μl of binding buffer [20 mM HEPES (pH 7.9), 5 mM MgCl2, 4 mM DTT, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 2 mM levamisol, 0.1 μg/ml aprotinin, 0.1 μg/ml bestatin, 2 μg poly(dIdC)]. The reaction mixtures were then loaded onto 6% native polyacrylamide gels in 0.09 M Tris borate, 2 mM DTT, 20% glycerol, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 2 mM levamisol, and stored in the dark before analysis at 4°C (generally within 24 h) and analyzed by chemiluminescence (Pierce, Rockford, IL).

Fig. 1. A, logarithmically growing K562 cells were exposed to the designated concentration of PD184352 for 48 h in the presence (●) or absence (○) of 200 nM of STI571, after which the percentage of apoptotic cells determined by morphological assessment of ≥1000 cells. B, cells were exposed to drugs as described in A, after which loss of MMP (Δψm) was assessed in DiOC6− stained specimens by flow cytometric analysis as described previously (32). C, K562 cells were exposed for 48 h to the designated concentration of STI571 in the presence (●) or absence (○) of 5 μM of PD184352, after which apoptosis was monitored as above. D, cells were treated as in C, after which the percentage of cells exhibiting loss of Δψm was determined as in B. In each case, values represent the means for three separate determinations; bars, ± SD.

RESULTS

Effects of STI571 and MEK1/2 Inhibitors on Mitochondrial Damage and Apoptosis in K562 Cells. To assess the effects of MEK/MAPK inhibition on STI571-induced apoptosis, K562 cells were exposed for 48 h to various concentrations of PD184352 in the presence or absence of 200 nM of STI571, after which the percentage of cells exhibiting the characteristic morphological features of cell death was determined. PD184352 by itself exerted minimal effects at concentrations ≤ 10 μM (percentage apoptosis < 3%), whereas 200 nM STI571 induced apoptosis in ~6% of cells (Fig. 1A). However, coadministration of PD184352 (1 μM) with STI571 resulted in a very substantial potentiation of apoptosis (e.g., to ~20%), with values exceeding 75% for PD184352 concentrations ≥ 5 μM. Roughly parallel results were obtained when loss of MMP (∆ψm) was monitored (Fig. 1B), although the loss of ∆ψm in PD184352-treated cells was somewhat greater than the extent of apoptosis.

Dose-response effects of STI571 (48 h) on apoptosis and mitochondrial damage in K562 cells were then examined in the presence or absence of PD184352.
absence of 5 μM PD184352 (Fig. 1, C and D). STI571 concentrations of 150–250 nM were minimally toxic to these cells but resulted in marked lethality when combined with PD184352 (Fig. 1C). The extent of apoptosis increased to ~20% in cells treated with 300 nM STI571 but approached 75% when PD184352 was coadministered. Finally, whereas 500 nM STI571 triggered apoptosis in ~50% of cells, values increased to >90% with PD184352. Similar results were noted when changes in MPP were monitored (Fig. 1D). Together, these findings indicate that PD184352 strikingly increases STI571-induced mitochondrial damage and apoptosis in K562 cells in a dose-dependent manner.

A time course analysis of cells exposed to 200 nM STI571 + 5 μM PD184352 revealed a modest increase in apoptosis after 24 h of cotreatment but a very substantial increase by 36 h (e.g., ~50%), with nearly 100% apoptotic cells at 72 h (Fig. 2A). Thus, essentially subtoxic concentrations of PD184352 dramatically increased STI571-induced mitochondrial damage and apoptosis in K562 cells, most notably at exposure intervals ≥36 h. Median dose effect analysis was used to characterize interactions between STI571 and PD184352 in regard to both induction of apoptosis and loss of ΔΨm (Fig. 2B). Combination index values significantly <1.0, corresponding to synergistic interactions, were obtained in each case. In addition, the MTS assay, which reflects both cell proliferation and viability, was used to assess interactions between very low concentrations of PD184352 (1.5 μM) and STI571 (50 nM). Whereas the drugs were nontoxic when given alone for 72 h, the combination reduced viability by >33% (Fig. 2C). Finally, coadministration of two other pharmacological MEK1/2 inhibitors (PD98059 or U0126), which by themselves were nontoxic, with STI571 (200 nM; 48 h) also resulted in a striking increase in apoptosis in K562 cells (Fig. 2D) consistent with a role for inhibition of MEK1/2 in enhanced lethality.

The potentiation of STI571-induced DNA damage in K562 cells by PD184352 is highlighted by the results of TUNEL assays depicted in Fig. 3. Treatment of K562 cells for 48 h with 200 nM of STI571 or 5 μM of PD184352 individually resulted in only a minimal degree of TUNEL positivity, reflecting cells containing DNA with free 3’-OH ends. In contrast, cells exposed to both agents exhibited a striking increase in the number of TUNEL-positive cells.

Interactions between STI571 and MEK1/2 Inhibitors in Other Hematopoietic Cell Types. Interactions between STI571 and PD184352 were then examined in other cell types (Fig. 4). The Bcr/Abl+ cell line LAMA 84 is somewhat more sensitive to STI571 than K562 cells (35). However, coadministration of 5 μM of PD184352 with 200 nM of STI571, both of which were only modestly toxic to LAMA 84 cells when given alone, resulted in a marked increase in the percentage of apoptotic cells after 36 h (e.g., ~70%). Parallel results were obtained when loss of ΔΨm was monitored (data not shown). In contrast, 200 nM of STI571 was nontoxic to Bcr/Abl- U937 cells, and cotreatment with PD184352 did not result in an increase in lethality. PD184352 alone exerted some toxicity toward HL-60 cells, as we have reported previously (23), but STI571 by itself did not induce apoptosis in these cells, nor did it enhance the lethal effects of PD184352. Finally, normal peripheral blood mononuclear cells were immune to the effects of these agents administered individually or together. Collectively, these findings suggest the synergism between STI571 and MEK1/2 inhibition is restricted to Bcr/Abl+ cells.

Effects of Caspase Inhibitors on Mitochondrial Damage and Activation of the Apoptotic Caspase Cascade by STI571/ PD184352 in K562 Cells. To confirm that potentiation of STI571-induced lethality by MEK1/2 inhibition reflected enhanced apoptosis, Western analysis was performed to monitor the expression of various proteins implicated in this process (Fig. 5). At the 24 or 48 h exposure intervals, STI571 or PD184352 administered alone exerted minimal effects on PARP degradation, procaspase 3 and 8 cleavage, or cytochrome c release. However, in cells exposed to the combination of STI571 and PD184352 for 24 h, PARP degradation, procaspase 3 and 8 activation, and cytosolic cytochrome c release could be faintly discerned, whereas at 48 h, each of these events was very pronounced. (Fig. 5B). A more detailed time course study revealed that a small amount of cytochrome c release was first noted at 24 h in STI571/ PD184352-treated cells but that extensive cytochrome c redistribution occurred at 36 h (Fig. 5C), consistent with the time course study of apoptotic morphology (Fig. 2A). To gain insights into the hierarchy of events accompanying cell death, cells were exposed to STI571 + PD184352 for 48 h in the presence or absence of the
general caspase inhibitor ZVAD-fmk or the caspase 8 inhibitor IETD-fmk (Fig. 6). It can be seen that the broad caspase inhibitor ZVAD-fmk was highly effective in blocking STI571/PD184352-induced apoptosis, whereas IETD-fmk was significantly less so (Fig. 6A). Parallel results were obtained when PARP degradation and procaspase 3 activation were monitored (Fig. 6B). This indicates that activation of the extrinsic, caspase 8-related pathway in all likelihood represents a secondary process in cells exposed to STI571 in combination with MEK1/2 inhibitors. However, neither caspase inhibitor blocked cytochrome c release, suggesting that redistribution of cytochrome c represents an upstream event in STI571/PD184352-mediated apoptosis.

**Effects of STI571/PD184352 on Expression of Antiapoptotic Proteins in K562 Cells.** Promotion of survival by the Bcr/Abl kinase has been linked to increased expression of antiapoptotic proteins, notably Bcl-xL (8). To determine what effect, if any, MEK1/2 inhibition might have on STI571-related perturbations in the expression of such proteins, Western analysis was used (Fig. 7). Administration of STI571(200 nm) or 5 μM PD184352 individually for 24 h resulted in small decreases in expression of Bcl-2 and Bcl-xL, but little change in XIAP or Mcl-1 protein levels (Fig. 7A). However, coadministration of these agents resulted in very marked declines in Bcl-2, Bcl-xL, and Mcl-1 protein levels, but no effect on XIAP. Moreover, whereas down-regulation of Bcl-2 expression in STI571/PD184352-treated cells was substantially diminished by ZVAD-fmk, suggesting that this process occurs secondary to caspase activation, down-regulation of Bcl-xL and Mcl-1 was not reduced, indicating that the latter events occur upstream of activation of the caspase cascade (Fig. 7B).

**PD184352 Promotes STI571-induced G0 G1 Arrest.** Flow cytometry was used to examine the effects of these agents on the cell

---

**Fig. 3.** K562 cells were exposed to either no drug (A), 5 μM PD184352 (B), 200 nm STI571 (C), or the combination of STI571 and PD184352 (D) for 48 h, after which cytospin preparations were obtained and stained with fluorescein-labeled dUTP in conjunction with terminal transferase (TUNEL assay) as described in “Materials and Methods.” Slides were viewed under fluorescence microscopy using a ×60 oil objective. Representative fields are shown; two additional studies yielded equivalent results.

**Fig. 4.** Cells were exposed to 200 nm STI571 ± 5 μM PD184352 for 36 or 48 h after which cytospin preparations were obtained, stained with Wright-Giemsa, and viewed under light microscopy. The percentage of apoptotic cells was determined by scoring ≥ 10 randomly selected fields encompassing ≥ 1000 cells. Values represent the means for three separate experiments performed in triplicate; bars, ± SD. A, LAMA 84 (36 h); B, U937; C, HL-60; D, normal peripheral blood mononuclear cells (all 48 h).
Methods.

Each lane was loaded with 25 μg protein; blots were stripped and reprobed for expression of cytochrome c. Each lane contained 25 μg of protein. The results of a representative study are shown; two additional experiments yielded equivalent results.

Coadministration of PD184352 Blocks STI571-associated ERK Activation and Promotes Bcr-Abl Down-Regulation, Phosphorylation of JNK and p38 MAPK, and Dephosphorylation of Stat5, CREB, and p34cdc2 in K562 Cells. Effects of combined treatment of K562 cells for 24 h with STI571 and PD184352 were then examined in relation to effects on various signal transduction pathways (Fig. 9).

Unexpectedly, levels of phospho-ERK were increased in STI571-treated cells at this time, whereas total ERK expression remained unchanged (Fig. 9A). As anticipated, activation (phosphorylation) of ERK was abrogated by coadministration of PD184352. Interestingly, exposure of cells to STI571 alone failed to induce phosphorylation (activation) of p38 MAPK and JNK, whereas both stress-related kinases were robustly activated in cells exposed to the combination of STI571 and PD184352. PD184352 alone modestly decreased phospho-CREB levels, an effect that was markedly potentiated by coadministration of STI571. STI alone induced a modest reduction in expression of phospho-Stat5, a downstream Bcr/ABL target, but com-

Fig. 6. K562 cells were exposed to 200 nM STI571 ± 5 μM PD184352 for 24 h in the presence or absence of ZVAD-fmk, after which proteins were isolated, separated by SDS-PAGE, and probed with antibodies directed against Bcl-2, Bcl-xL, XIAP, or Mcl-1. Each lane contained 25 μg protein. B, K562 cells were exposed to 200 nM STI571 ± 5 μM PD184352 for 24 h in the presence or absence of 20 μM ZVAD-fmk, after which proteins were separated as above (25 μg protein per lane) and probed with antibodies to Bcl-2, Bcl-xL, XIAP, or Mcl-1. Because of low levels of Bcl-2 expression in K562 cells, dilutions of Bcl-2 antibodies were 1:200 versus 1:1000 for the other antibodies. In each case, blots were stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer. The results of a representative study are shown; two other experiments yielded equivalent results.

Fig. 7. A, K562 cells were exposed to 200 nM STI571 ± 5 μM PD184352 for 24 h, after which the cells were lysed, the proteins separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies directed against Bcl-2, Bcl-xL, Mcl-1, or XIAP. Each lane contained 25 μg protein. B, K562 cells were exposed to 200 nM STI571 ± 5 μM PD184352 for 24 h in the presence of 10 μM IETD-fmk, after which proteins were separated as above (25 μg protein per lane) and probed with antibodies to Bcl-2, Bcl-xL, XIAP, or Mcl-1. Because of low levels of Bcl-2 expression in K562 cells, dilutions of Bcl-2 antibodies were 1:200 versus 1:1000 for the other antibodies. In each case, blots were stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer. The results of a representative study are shown; two other experiments yielded equivalent results.

cycle distribution of K562 cells at 24 h (Fig. 8), a point time shortly preceding the extensive induction of apoptosis accompanying combined drug treatment. PD184352 by itself exerted minimal effects on cell cycle distribution. STI571 (200 nM) modestly increased the G0/G1 and G2/M fractions, and decreased the S phase fraction. In contrast, combined exposure to STI571 and PD184352 resulted in a substantial increase in the G0/G1 population (e.g., to ~65%), which was significantly greater than that observed in cells exposed to STI571 alone (P < 0.005). On the other hand, the decline in the S phase fraction was not significantly greater than that noted in STI571-treated cells (P > 0.05). However, STI571/PD184352-treated cells exhibited a reduction rather than an increase in the percentage of cells in G2/M (P < 0.002 versus STI571 alone). These findings indicate that MEK1/2 inhibition potentiates the ability of STI571 to induce G0/G1 arrest in K562 cells.

Fig. 5. K562 cells were exposed to 200 nM STI571 ± 5 μM PD184352 for 24 (A) or 48 (B) h, after which cell pellets obtained and protein subjected to Western analysis as described in “Materials and Methods.” After separation on SDS-PAGE gels, blots were probed with antibodies directed against PARP, pro-caspase 3, pro-caspase 3 cleavage products, and pro-caspase 8. Each lane was loaded with 25 μg of protein, and after analysis, blots were stripped and reprobed for tubulin to ensure equal loading and transfer of protein. Alternatively, S-100 cytosolic fractions were obtained as described in “Materials and Methods” and probed for expression of cytochrome c. Each lane contained 25 μg of protein. The results of a representative experiment are shown; two additional experiments yielded equivalent results.
bination with PD184352 resulted in an even more pronounced decline in Stat5 phosphorylation. The combination of STI571 and PD184352 also resulted in a striking increase in p34<sup>cdk2</sup> activation (dephosphorylation), whereas STI571 alone exerted only a minimal effect. Interestingly, STI571 alone failed to reduce levels of the M<sub>C</sub> 210,000 Bcr-Abl protein, whereas expression was barely detectable in cells exposed to both PD184352 and STI571. In separate studies, phospho-(tyrosine)-Bcr-Abl levels were diminished in cells exposed to STI571 and, as anticipated given the extensive reduction in Bcr/Abl protein levels, were essentially undetectable in cells treated with the combination of STI571 and PD184352 (data not shown). However, whereas treatment with STI571 alone (200 nM) significantly reduced Akt activity in K562 cells (e.g., by 42 ± 7%), this decline was not additionally enhanced by coadministration of PD184352 (e.g., 29 ± 0.9%; P > 0.05; data not shown).

EMSA analysis was used to document a reduction in Stat5 DNA binding in STI571/MEK1/2 inhibitor-treated cells (Fig. 9B). Coadministration of 10 μM of U0126 alone had little effect on DNA binding to a Stat5 consensus oligonucleotide in the gel shift assay, whereas 200 nM of STI571 modestly reduced binding. However, combined treatment of cells with both STI571 and U0126 or PD184352 resulted in a marked reduction in the association of Stat5 and the labeled probe. Essentially identical results were obtained when 5 μM of PD184352 was used instead of U0126 (data not shown).

In view of evidence that apoptotic caspases can induce cleavage of various signaling proteins (36), parallel studies were performed in STI571/MEK1/2 inhibitor-treated cells (Fig. 9D). Exposure of K562 cells to STI571 concentration of 1 μM alone for 48 h resulted in a marked reduction in Bcr/Abl levels, an effect that was blocked by the caspase-3 inhibitor DEVD-fmk and the pan-caspase inhibitor Boc-fmk (25 μM each). Taken together with the preceding findings, these observations suggest that MEK1/2 inhibition enhances the ability of STI571 to promote the caspase-3-mediated cleavage of the Bcr/Abl protein.

Treatment of K562 Cells with STI571 Initially Inhibits and Subsequently Promotes ERK1/2 Phosphorylation, an Effect That Is Abrogated by Coadministration of PD184352. To additionally characterize the effects of STI571 and PD184352 on MAPK activation, a more detailed time course analysis was carried out (Fig. 10). In these studies, ratios of expression of activated (phosphorylated) p42/44 MAPK versus total MAPK were determined by densitometric analysis of Western blots over a 48-h exposure interval (Fig. 10A). Western blots depicting phosphorylation of MAPK at 6 and 24 h are also shown in Fig. 10B. Interestingly, at the 6-h interval, 200 nM of STI571 significantly reduced MAPK activation, consistent with results of previous reports (12). However, at later intervals (e.g., 24 and 36 h), STI571-treated cells displayed a significant increase in MAPK activation (Fig. 10A). By 48 h, MAPK activation had declined to control levels. In all of the cases, coadministration of PD184352 essentially abrogated phospho-MAPK expression. To rule out the possibility that activation of MAPK occurs only in response to a low concentration of STI571, phospho-ERK1/2 expression was monitored in K562 cells exposed to 2 μM of STI571 for 24 h. As in the case of lower drug concentrations, treatment with 2 μM of STI571 resulted in clear evidence of enhanced ERK1/2 phosphorylation/activation (Fig. 10C), an effect that was prevented by PD184352 (data not shown).
Activation of p38 MAPK Contributes to STI571/PD184352-induced Lethality in K562 Cells. To assess the functional significance of enhanced phosphorylation (activation) of p38 MAPK in STI571/PD184352-induced lethality, K562 cells were exposed for 48 h to 200 nM of STI571 and 10 μM of PD184352 in the presence or absence of SB203580 or SB202190, two pharmacological inhibitors of the p38 MAPK. As shown in Fig. 10D, coadministration of either p38 MAPK inhibitor (10 μM each) partially but significantly reduced the extent of apoptosis in STI571/PD184352-treated cells (P < 0.01) versus STI571 + PD184352 alone. These findings suggest that the reciprocal activation of p38 MAPK contributes to potentiation of STI571-related lethality by MEK1/2 inhibition.

K562R Cells Display Increased Basal Bcr-Abl and Phospho-ERK1/2 Expression and Are Resistant to STI571-induced Apoptosis. An attempt was then made to characterize interactions between MEK inhibitors and STI571 in Bcr/Abl-positive cells resistant to the latter agent. To this end, a multidrug-resistant K562 cell line (K562R) characterized primarily, isolated by culturing cells in progressively higher concentrations of doxorubicin, was used (30). Unexpectedly, K562R cells expressed an ~3-fold increase in Bcr/Abl protein levels relative to the parental line (K562S) and a slightly greater relative increase in the expression of phospho-MAPK (Fig. 11A). In addition, basal Akt activity was increased by a factor of 1.9 ± 0.2 in K562R cells compared with controls (data not shown). A dose-response study revealed that K562R cells were substantially more resistant to STI571-induced apoptosis than the wild-type line, with an ~10-fold higher IC50 value (Fig. 11B).

PD184352 but not Verapamil Substantially Increases STI571-induced Mitochondrial Damage and Apoptosis in K562R Cells. As shown in Fig. 12A, a pharmacologically achievable STI571 concentration (e.g., 1.5 μM) was largely ineffective in inducing apoptosis in K562R cells. However, when 10 μM of PD184352, which was nontoxic to these cells, was coadministered, the extent of apoptosis in the resistant cells increased dramatically (e.g., to ~60%). Interestingly, verapamil (2 μM), which has been shown previously to increase the susceptibility of multidrug-resistant LAMA 84 cells to STI571 (35), was ineffective in the K562R line. In separate studies, coadministration of this concentration of verapamil increased by 5-fold the extent of apoptosis induced by 10 μM of doxorubicin (data not shown). These findings suggest that factors other than or in addition to alterations in drug uptake are involved in potentiation of STI571 lethality by PD184352. The shift in the STI571 dose-response curve after treatment of resistant cells with a nontoxic concentration of PD184352 is shown in Fig. 12B. Finally, whereas 1.5 μM STI571 alone minimally induced PARP degradation, procaspase-3 cleavage,
and cytochrome c release in K562R cells, addition of PD184352, which was inactive by itself, resulted in a marked potentiation of mitochondrial damage and caspase activation (Fig. 12 C). Together, these findings indicate that MEK1/2 inhibition is able to reverse, at least in part, STI571 resistance in leukemic cells overexpressing the Bcr/Abl protein.

**PD184352 Potentiates STI571-mediated Lethality in K562 Cells Specifically Developed for Resistance to STI571.** To determine whether MEK1/2 inhibitors could potentiate STI571-related lethality in Bcr/Abl+ cells specifically developed for resistance to STI571, a resistant K562 cell line was used that had been subcultured in progressively higher concentrations of STI571. As shown in the inset in Fig. 13A, these cells, designated K562-R-STI, exhibited a greater relative increase in Bcr/Abl protein expression compared with parental cells (e.g., ~6-fold) than the K562R cell line. A time course study (Fig. 13A) revealed that K562-R-STI cells were for all practical purposes immune to apoptosis induced by 2 μM of STI571 over a 72-h interval, whereas essentially 100% of parental cells were apoptotic after 48 h of drug exposure. Whereas 10 μM of PD184352 by itself exerted modest toxicity toward K562-R-STI cells (i.e., ~9% apoptotic), combination with a nontoxic concentration of STI571 (2 μM) for 48 h resulted in a very marked increase in cell death (~40% apoptotic; Fig. 13B). This finding indicates that MEK1/2 inhibitors can enhance the lethal effects of STI571 in Bcr/Abl+ cells developed specifically for resistance to this agent.

**DISCUSSION**

The present findings indicate that multiple pharmacological inhibitors of the MEK/MAPK pathway interact in a highly synergistic manner with STI571 to induce apoptosis in Bcr/Abl+ human myeloid leukemia cells. Whereas the ability of Bcr/Abl tyrosine kinase inhibi-
itors was predicted to overcome the growth advantage of Bcr/Abl cells, the striking capacity of such agents to induce apoptosis in leukemic progenitors was unanticipated and suggest that such cells depend on expression of this kinase for survival as well as enhanced proliferation. Although initial clinical results in CML with STI571 are extremely encouraging (13), the development of drug resistance and the more limited responsiveness of patients with blast crisis and Bcr/Abl– acute lymphoblastic leukemia represent persistent challenges (37). Such considerations have prompted attempts to circumvent resistance by developing strategies combining STI571 with other agents active against Bcr/Abl+ cells. For example, potentiation of apoptosis in such cells has been observed after combined exposure to STI571 and tumor necrosis factor-related apoptosis-inducing ligand (38) or the tyrosine kinase inhibitor AG490 (39). In addition, multiple groups have found that combination of STI571 with standard chemotherapeutic agents results in enhanced lethality toward Bcr/Abl-expressing cells, which in some cases may be synergistic (13, 14, 40, 41). Enhanced lethality has also been observed in Bcr/Abl-expressing cells exposed to STI571 in combination with arsenic trioxide (42). Recently, Vigneri and Wang (16) reported that the nuclear transport inhibitor leptomycin increased the lethality of STI571 toward Bcr/Abl+ cells through a mechanism that involved nuclear trapping of the Bcr/Abl protein. However, the ultimate effectiveness of this strategy in the context of pharmacologically achievable STI571 concentrations remains to be determined.

Interactions between MEK1/2 inhibitors and STI571 were noteworthy in several respects, including the extent of synergism, particularly at low drug concentrations, as well as the observed activity against otherwise STI571-resistant cells. Whereas activation of the MEK/MAPK module has generally been associated with antiapoptotic actions (19), the relationship between MEK/MAPK induction and Bcr/Abl expression is unclear. For example, MAPK has been reported to be a downstream target of the Bcr/Abl kinase (26), although Morgan et al. (43) could find no clear relationship between Bcr/Abl expression and MAPK activation in a panel of human leukemic cell lines. On the other hand, Kang et al. (29) reported that interruption of MEK1/2 (e.g., by PD98050) was a potent inducer of apoptosis in the K562 line, suggesting an important role for the MAPK cascade in the survival of Bcr/Abl+positive cells. Similarly, Woessmann and Miveschi (44) observed that disruption of ERK signaling, either by transfection with a dominant-negative ERK1 mutant or treatment with the MEK1/2 inhibitor UO126, induced apoptosis in K562 cells. Consistent with the results of Dan et al. (12), we also found that exposure of Bcr/Abl+ cells to STI571 resulted in down-regulation of activated MAPK, at least at early time points (e.g., 6 h). However, at later intervals, a significant increase in MAPK activation was observed in STI571-treated cells, an effect that was abrogated by addition of MEK1/2 inhibitors. Consequently, it is tempting to speculate that disruption of the Bcr/Abl pathway (e.g., by STI571) represents a stress for such cells, and in so doing elicits a compensatory cytoprotective MEK/MAPK response, which may operate, at least in part, through a Bcr/Abl-independent mechanism. Moreover, interference with the latter process may lead, through an as yet to be defined mechanism, to mitochondrial damage (e.g., cytochrome c release, loss of ΔΨm) and subsequent activation of the apoptotic cascade. However, the present results do differ from those of Oetzel et al. (45) who reported that the MEK1/2 inhibitor PD98059 failed to enhance STI571 lethality in murine pro-B lymphocytic Baf-1 cells transfected with a p185 Bcr/Abl construct. This discrepancy may stem from intrinsic differences in the responses of Bcr/Abl+ murine and human hematopoietic cells to interruption of the MEK/MAPK cascade. Alternatively, such differences might reflect the fact that lethality was examined at relatively early time intervals in the latter study (e.g., ≤ 24 h). In this regard, cell death induced by the combination of STI571 and PD184352 was also quite limited in K562 cells after a 24-h drug treatment, consistent with the results of Oetzel et al. (45) but increased dramatically at exposure intervals ≥ 36 h. In any case, it is apparent that the lethal consequences of combined Bcr/Abl kinase and MEK1/2 inhibition in K562 and LAMA 84 cells result from relatively late rather than early events.

Aside from the MEK1/2/MAPK pathway, several Bcr/Abl downstream targets have been postulated to promote survival of Bcr/Abl+ cells, including Bcl-xL, nuclear factor κB, and Akt, among others (8, 9, 46). It is possible that STI571 acts, at least in part, by down-regulating expression of these putative prosurvival proteins. This phenomenon may occur either through direct inhibition of the Bcr/Abl kinase or indirectly through cleavage of target proteins by apoptotic caspses. In this regard, it is noteworthy that the combination of STI571 + PD184352 (or STI571 alone) inhibited phosphorylation/activation of Bcr/Abl as anticipated (9) but also resulted in reduced levels of Bcr/Abl protein through a caspase-dependent process. Whereas exposure of Bcr/Abl+ cells to STI571 has not in general been associated with down-regulation of the Bcr/Abl protein, several other agents have been shown to act in this way, including arsenic trioxide (42, 47), geldanamycin (48), proteasome inhibitors (49), and the kinase inhibitor AG957 (50). Although reduction in Bcr/Abl protein expression by STI571/PD184352 was sensitive to the general caspase inhibitor ZVAD as well as the caspase-3 inhibitor DEVd, the possibility that proteosomal degradation may also be involved in this process cannot be excluded. Whatever the mechanism, the present findings raise the possibility that coadministration of MEK1/2 inhibitors with a low concentration of STI571 triggers an amplification loop in which Bcr/Abl protein expression is diminished through a caspase-dependent process. On the other hand, our inability to detect an additional reduction in Akt activity in cells exposed to the combination of STI571 and PD184352 argues against a contribution of this pathway to enhanced lethality.

Analogously, the combination of PD184352 and STI571 resulted in caspase-dependent down-regulation of the antiapoptotic protein Bcl-2. Cleavage of Bcl-2 into a proapoptotic fragment during the course of apoptosis has been reported previously (51) and may serve to ensure that the apoptotic process, once initiated, proceeds to completion. Coadministration of PD184352 with STI571 also resulted in a marked reduction in protein expression of Bcl-xL and Mcl-1 compared with the effects of STI571 alone, although the insensitivity of these events to ZVAD-fmk, in contrast to Bcl-2 down-regulation, suggests a primary mechanism of action. The finding that STI571/PD184352 treatment was associated with reduced levels of Mcl-1 is also consistent with previous reports demonstrating a requirement for MEK/MAPK activation in sustained expression of this antiapoptotic protein (52). In addition, it is important to note that Bcr/Abl lies upstream of the Stat family of transcription factors, which have been implicated in the regulation of antiapoptotic proteins such as Bcl-XL and Mcl-1 in hematopoietic cells (53–55). The observation that MEK1/2 inhibition enhanced STI571-mediated attenuation of Stat5 phosphorylation and DNA binding is consistent with such a model. Collectively, these findings suggest that combined inhibition of the MEK/MAPK and Bcr/Abl pathways results in diminished expression of multiple antiapoptotic proteins through both caspase-dependent and -independent mechanisms, which together may serve to amplify the cell death process.

Combined exposure of K562 cells to STI571 and PD184352 resulted in perturbations in other signaling cascades, although the functional contribution of these events to lethality remains to be determined. For example, cells exposed to both agents but not each agent individually exhibited a marked activation of both JNK and p38.
MAPK. However, whereas activation of such stress-related pathways have been linked to cell death in some systems (19), in others (23), including K562 cells (29), their role in cell death may be less critical than that of inhibition of MEK/MAPK. Nevertheless, the finding that the p38 MAPK inhibitors SB203580 and SB202190 partially attenuated STI571/PD184352-mediated apoptosis suggests a functional role for activation of this stress pathway in the lethal actions of this drug combination. Treatment of K562 cells with the combination of STI571 and PD184352 also resulted in diminished phosphorylation of CREB and p34^cdc2, both of which have been associated with promotion of apoptosis (56, 57). Elucidation of the functional role of these events, as well as that of JNK activation, in STI571/MEK2 inhibitor-related apoptosis awaits additional analysis, and studies designed to address this issue are currently in progress.

Resistance of Bcr/Abl+ cells to STI571 may occur via multiple mechanisms, including amplification of the Bcr/Abl gene, mutations in the Bcr/Abl kinase ATP binding site, or reduction in intracellular drug uptake, among others (35, 58–60). Although gene amplification is not universally encountered in resistant cells, increases in Bcr/Abl protein expression commonly occurs, at least in cultured cell lines (58, 60). The K562R line was originally developed by subculturing cells in progressively higher concentration of doxorubicin and was initially characterized as an overexpressor of the Pgp protein (30). However, in view of the ability of the Bcr/Abl protein to confer resistance to multiple cytotoxic drugs (7), it is not surprising that such cells would in addition exhibit increased Bcr/Abl protein expression. K562R cells also displayed a significant increase in basal MAPK activity, consistent with the notion that MAPK is a downstream target of the Bcr/Abl kinase (28, 29). The degree of resistance of K562R cells to STI571-mediated lethality is similar to if not greater than that exhibited by lines described previously in the literature (35, 58, 59). Significantly, coadministration of a nontoxic concentration of PD184352 with a pharmacologically achievable concentration of STI571 (e.g., 1.5 µM) resulted in a substantial increase in mitochondrial damage (e.g., cytochrome c release), caspase activation, and apoptosis in otherwise resistant K562 cells. In fact, the response of resistant cells to the combination was comparable with that of sensitive cells exposed to STI571 alone. In contrast to the results of Mahon et al. (35) who found that STI571-resistant LAMA 84 cells exhibited overexpression of Bcr/Abl and Pgp, and that verapamil increased STI571 sensitivity, coadministration of verapamil had a negligible effect on STI571-resistant LAMA 84 cells. In contrast to the results of Mahon et al., the STI571-resistant K562R line was developed by subculturing cells in progressively higher concentration of doxorubicin and was initially characterized as an overexpressor of the Pgp protein. However, in view of the ability of the Bcr/Abl protein to confer resistance to multiple cytotoxic drugs, it is not surprising that such cells would in addition exhibit increased Bcr/Abl protein expression. K562R cells also displayed a significant increase in basal MAPK activity, consistent with the notion that MAPK is a downstream target of the Bcr/Abl kinase (28, 29).

To address this issue are currently in progress. However, an alternative approach would be to administer STI571 in conjunction with a MEK1/2 inhibitor such as PD184352, which is capable of inhibiting MAPK activation in tumor cells in mice (22) and which is currently entering clinical trials in humans. Thus, if 90% of STI571 were bound to α1-acidic glycoprotein or other plasma proteins and presumably inactive, free concentrations of 100–200 nm might, under conditions of MEK/MAPK inhibition, be sufficient to kill Bcr/Abl+ leukemic cells, as suggested by the present findings. Pending results of ongoing Phase I trials of MEK/MAPK inhibitors in humans, it would clearly be desirable to test this strategy in an animal model system. Accordingly, plans to examine the in vivo potential of this approach are under development.
APOPOTOSIS IN HUMAN LEUKEMIA CELLS


Pharmacologic Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase/Mitogen-activated Protein Kinase Inhibitors Interact Synergistically with STI571 to Induce Apoptosis in Bcr/Abl-expressing Human Leukemia Cells

Chunrong Yu, Geoffrey Krystal, Lyuba Varticovksi, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/62/1/188

Cited articles
This article cites 59 articles, 43 of which you can access for free at:
http://cancerres.aacrjournals.org/content/62/1/188.full#ref-list-1

Citing articles
This article has been cited by 51 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/62/1/188.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.