

Flavopiridol Potentiates STI571-induced Mitochondrial Damage and Apoptosis in BCR-ABL-positive Human Leukemia Cells¹

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ABSTRACT

Purpose: The goal of this study was to characterize interactions between the Bcr/Abl kinase inhibitor STI571 and the cyclin-dependent kinase inhibitor flavopiridol in Bcr/Abl⁺ human leukemia cells.

Experimental Design: K562 leukemia cells were exposed to STI571 ± flavopiridol for 24 or 48 h, after which mitochondrial damage, caspase activation, expression/activation of signaling and cell cycle regulatory proteins, and apoptosis were assessed.

Results: In K562 cells, coadministration of marginally toxic concentrations of STI571 (200 nM) and flavopiridol (150 nM) for 48 h resulted in a marked increase in mitochondrial damage (e.g., cytochrome *c* release), activation of caspase-3, caspase-8, and Bid, and apoptosis. Similar interactions were observed in Bcr/Abl⁺ LAMA-84 cells but not in leukemic cells that fail to express Bcr/Abl (e.g., HL-60, U937, Jurkat). STI571/flavopiridol-mediated apoptosis was associated with the caspase-independent down-regulation of Bcl-x_L and Mcl-1, activation of extracellular signal-regulated kinase and c-Jun NH₂-terminal kinase, and the caspase-dependent release of Smac/DIABLO and loss of Δψ_m. Coadministration of flavopiridol and STI571 did not result in changes in levels of expression of Bcl-2, phospho-Stat5, phospho-p34^{cdc2}, or Bcr/Abl. Finally, STI571/flavopiridol effectively induced apoptosis in STI571-resistant K562 cells displaying amplification of the Bcr/Abl protein.

Conclusions: Together, these findings indicate that the cyclin-dependent kinase inhibitor flavopiridol induces mul-

multiple perturbations in signaling pathways in STI571-treated Bcr/Abl⁺ human leukemia cells that culminate in mitochondrial injury, caspase activation, and apoptosis. They also suggest that simultaneous disruption of survival signaling and cell cycle regulatory pathways may represent an effective strategy in Bcr/Abl⁺ malignancies.

INTRODUCTION

The Bcr/Abl oncogene encodes a fusion protein that is found in the cells of 95% of patients with CML³ (1). Constitutive activation of the Bcr/Abl tyrosine kinase confers a survival advantage to hematopoietic cells, contributing to leukemic transformation (2). More specifically, expression of the Bcr/Abl kinase renders cells relatively insensitive to apoptosis induced by diverse stimuli, including growth factor deprivation and cytotoxic drugs (3, 4). Although the downstream targets of Bcr/Abl responsible for apoptosis resistance are not known with certainty, multiple signaling/survival pathways have been implicated in this phenomenon, including those related to nuclear factor κB, Stat5, MEK/MAP kinase, Bcl-x_L, and Akt, among others (5–9). Recently, considerable attention has focused on STI571, a tyrosine kinase inhibitor that inhibits the Bcr/Abl c-Kit, and to a lesser extent, other kinases (10). STI571 has been shown to inhibit the growth of and induce apoptosis in Bcr/Abl⁺ leukemia cells *in vitro* (11, 12). Significantly, oral administration of STI571 to patients with CML results in clinical responses in the large majority of patients (13). However, the emergence of STI571 resistance in CML patients initially responsive to this agent (14) has prompted the search for alternative approaches to the treatment of this disease.

Flavopiridol (L86–8275, HMR 1275) is a semisynthetic flavone, derived from the flavonoid rohitukine, that acts as potent inhibitor of multiple CDKs, including CDK1, CDK2, CDK4/6, and CDK7 (15). It is the first of the pharmacological CDK inhibitors to enter clinical trials in humans (16). As might be anticipated from its mechanism of action, flavopiridol is a potent inhibitor of cell cycle traverse, triggering cell cycle arrest in G₁-S as well as in G₂-M (17). Interestingly, flavopiridol is also an effective inducer of apoptosis, particularly in malignant hematopoietic cells (18). Flavopiridol has been shown to potentiate the lethality of conventional cytotoxic drugs in a sequence-dependent manner (19). Whether such actions stem from inhibition of CDKs or involve other flavopiridol actions remain to be determined.

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³ The abbreviations used are: CML, chronic myelogenous leukemia; MEK, MAP/ERK kinase; MAP, mitogen-activated protein; MAPK, mitogen-activated protein kinase; CDK, cyclin-dependent kinase; JNK, c-Jun NH₂-terminal kinase; ERK, extracellular signal-regulated kinase; Smac, second mitochondria-derived activator of caspases; DIABLO, direct IAP-binding protein with low pI; CREB, camp response element-binding protein; PARP, poly(ADP-ribose) polymerase.

Currently, little information is available concerning the effects of simultaneous interruption of survival signaling and cell cycle regulatory pathways with respect to neoplastic cell behavior. In this context, we have recently reported that combined exposure of multiple human leukemia cell lines to various MEK1/2 inhibitors in conjunction with UCN-01 (7-hydroxystaurosporine), an inhibitor of Chk1 as well as several CDKs (20), resulted in a dramatic potentiation of mitochondrial damage (e.g., cytochrome *c* release) and apoptosis (21). Based upon these findings, the possibility that flavopiridol might enhance the lethal actions of an agent such as STI571 appeared plausible. To test this hypothesis, we have examined interactions between STI571 and flavopiridol in Bcr/Abl⁺ leukemia cells in relation to mitochondrial damage, caspase activation, signaling events, and apoptosis. Here we report that flavopiridol interacts with STI571 in a highly synergistic manner to trigger mitochondrial injury and apoptosis in such cells, including those resistant to STI571 and displaying Bcr/Abl amplification. Together, these findings suggest that combining cell cycle inhibitors with kinase inhibitors such as STI571 that target specific survival signaling pathways may represent a novel and effective antileukemic strategy.

MATERIALS AND METHODS

Cells. K562, U937, HL-60, and Jurkat human leukemia cells were purchased from American Type Culture Collection (Manassas, VA). 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). Drug-resistant K562 cells (K562R) were derived from the parental line by subculturing in progressively higher concentrations of doxorubicin as described previously (22). They were cultured in drug-free medium before all experimental procedures.

Reagents. STI571 was kindly provided by Dr. Elizabeth Buchdunger (Novartis Pharmaceuticals, Basel, Switzerland) and prepared as a 10 mM stock solution in sterile DMSO (Sigma, St. Louis, MO). Flavopiridol was kindly provided by Dr. Edward Sausville (National Cancer Institute/Cancer Therapy Evaluation Program, Bethesda, MD), stored as a dry powder at 4°C, and formulated in sterile water before use. Boc-fmk was purchased from Enzyme Products, Ltd. (Livermore, CA), and formulated in sterile DMSO before use.

Assessment of Apoptosis. After drug exposures, cyto-centrifuge preparations were stained with Wright-Giemsa and viewed by light microscopy to evaluate the extent of apoptosis as described previously (23). Essentially equivalent results were obtained when apoptosis was monitored by Annexin V/PI staining and flow cytometric analysis.

Determination of Mitochondrial Membrane Potential ($\Delta\Psi_m$). Mitochondrial membrane potential was monitored by measuring uptake of 3,3-dihexyloxycarbocyanine iodide using a FACScan flow cytometer and CellQuest software (Becton Dickinson and Immunocytometry Systems, San Jose, CA) as described previously (23).

Preparation of S-100 Fractions and Assessment of Cytochrome *c* and Smac/DIABLO Release. Cells were harvested after drug treatment and the cytosolic S-100 fraction isolated by ultracentrifugation as we have described previously in detail (24). The extent of cytochrome *c* and Smac-DIABLO release in the S-100 fractions was determined by Western analysis as described below.

Western Analysis. Isolation and processing of proteins was carried out as described previously (24). The source and dilution of antibodies were as follows: Bcl-2 1:1000, mouse monoclonal (Dako, Carpinteria, CA, and Santa Cruz Biotechnology, Santa Cruz, CA); Bcl-x_L 1:1000, rabbit polyclonal (Santa Cruz Biotechnology); XIAP 1:1000, rabbit polyclonal (R&D Systems, Minneapolis, MN); Mcl-1 1:1000, mouse monoclonal (Pharminigen, 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 and phospho-Stat5 1:1000, rabbit polyclonal (Cell Signaling Technology); phospho-cdc2 1:1000, rabbit polyclonal (Cell Signaling Technology); phospho-CREB 1:1000, rabbit polyclonal; Smac/DIABLO 1:1000, rabbit polyclonal (Upstate Biotechnology, Lake Placid, NY); c-Abl (24–11) 1:1000, mouse monoclonal (Santa Cruz Biotechnology); pRb and cyclin D₁ 1:1000, mouse monoclonal (Pharminigen); cleaved caspase-9 1:1000, rabbit polyclonal (Cell Signaling Technology); PARP (C-2–10) 1:3000, mouse monoclonal (BioMol Research Laboratories, Plymouth, MA); procaspase-3 1:1000, mouse monoclonal (Transduction Laboratories); cytochrome *c* 1:500, mouse monoclonal; caspase 8 1:1000, rabbit polyclonal (Pharminigen); and α -tubulin 1:2000 (Calbiochem). Blots were washed three times at 5 min each in PBS-0.2% Tween 20 and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories, Hercules, CA) for 1 h at 22°C. Blots were again washed three times at 5 min each in PBS-0.2% Tween 20 and then developed by enhanced chemiluminescence (Pierce, Rockford, IL).

Cell Cycle Analysis. After treatment, cells were pelleted and fixed in 70% ethanol on ice for 1 h and resuspended in 1 ml of cell cycle buffer (0.38 mM sodium citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml propidium iodide; all from Sigma) at a concentration of 10⁶ cells/ml. Cell cycle analysis was carried out using a FACScan flow cytometer and CellQuest software as described previously (23).

Statistical Analysis. The significance of differences between experimental conditions was determined using the two-tailed student *t* test. Drug interactions were examined for synergism or antagonism using Median Dose Effect analysis (Calcsyn; Biosoft, Ferguson, MO).

RESULTS

Exposure of K562 cells for 48 h to STI571 concentrations <300 nM weakly induced apoptosis, whereas concentrations of 300–500 nM induced lethality in 20–50% of cells (Fig. 1A).

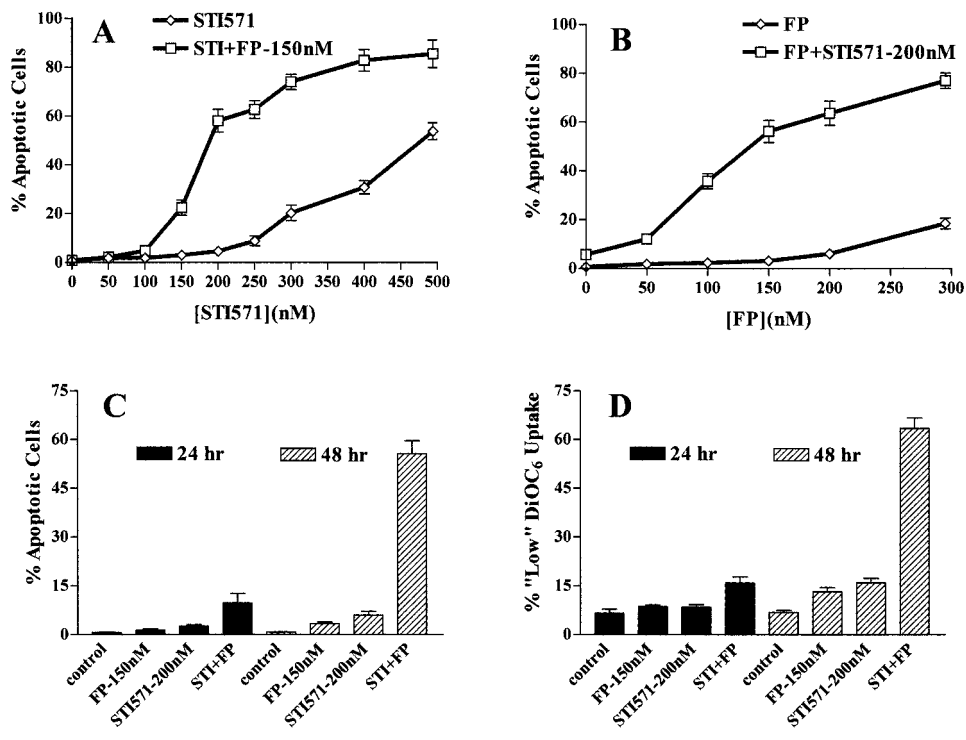


Fig. 1 Coexposure of K562 cells to STI571 and flavopiridol results in a marked potentiation of mitochondrial damage and apoptosis. *A*, K562 cells were exposed for 48 h to 150 nM FP and the designated concentration of STI, after which the percentage of apoptotic cells was determined by morphological assessment of Wright Giemsa-stained slides as described in "Materials and Methods." *B*, K562 cells were exposed for 48 h to 200 nM STI571 and the designated concentration of FP, after which apoptosis was monitored as above. Alternatively, K562 cells were exposed to 150 nM FP \pm 200 nM STI for 24 or 48 h, after which the percentage of apoptotic cells (*C*) or cells exhibiting low uptake of 3,3'-dihexyloxacarbocyanine iodide (DiOC₆), reflecting loss of $\Delta\psi_m$, (*D*) determined as in "Materials and Methods." Values represent the means for three separate experiments performed in triplicate \pm SD. FP, flavopiridol; STI, STI571.

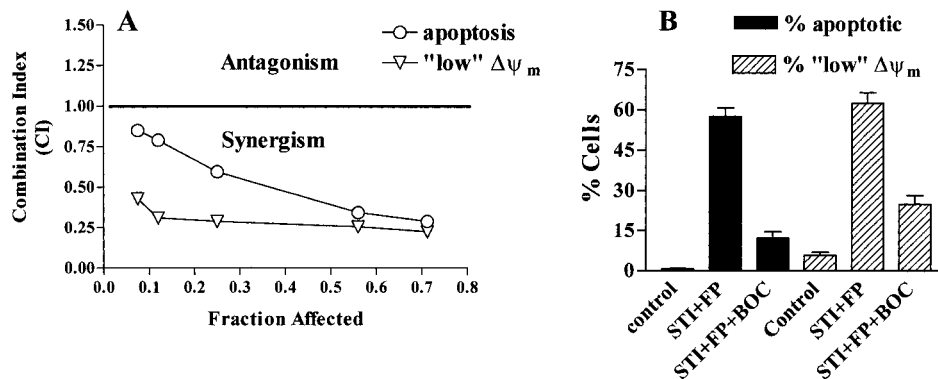


Fig. 2 Synergistic induction of apoptosis by STI571/flavopiridol is largely blocked by the pan-caspase inhibitor Boc-fmk, whereas loss of $\Delta\psi_m$ is partially attenuated. *A*, K562 cells were exposed to various concentrations of FP and STI at a fixed ratio (1:1.33) for 48 h, after which the percentage of apoptotic cells or cells displaying a decline in $\Delta\psi_m$ was monitored as described in "Materials and Methods." The combination index was determined in relation to the fraction of cells affected using Median Dose Effect analysis. Results are representative of three separate experiments. *B*, K562 cells were exposed for 48 h to 200 nM STI + 150 nM FP in the presence or absence of 25 μ M BOC, after which the percentage of apoptotic cells (*left*; \blacksquare) or cells exhibiting a decline in $\Delta\psi_m$ (*right*; \hatched) determined as described in "Materials and Methods." Values represent the means for three separate experiments performed in triplicate \pm SD. STI, STI571; FP, flavopiridol; BOC, Boc-fmk.

However, coadministration of 150 nM flavopiridol, which was essentially nontoxic by itself (*i.e.*, inducing <5% cell death), dramatically increased apoptosis in cells exposed to STI571 concentrations \geq 150 nM. As shown in Fig. 1*B*, coadministration of flavopiridol at concentrations \geq 100 nM markedly potentiated the lethal effects of 200 nM STI571, and these effects were particularly pronounced in the case of 300 nM flavopiridol. The lethal effects of 200 nM STI571 + 150 nM flavopiridol, reflected by the appearance of apoptotic cells (Fig. 1*C*) or loss of $\Delta\psi_m$

(Fig. 1*D*), were modest after 24 h but very extensive after 48 h, indicating a delayed mode of action for this drug combination. Concordant results were obtained when 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2*H*-tetrazolium dye reduction was used as an end point (data not shown).

Median Dose Effect analysis was used to characterize interactions between STI571 and flavopiridol with respect to both induction of apoptosis and loss of $\Delta\psi_m$ at 48 h (Fig. 2*A*). Combination index values < 1.0 were consistently obtained,

Fig. 3 Flavopiridol potentiates STI571-mediated lethality only in Bcr/Abl⁺ leukemic cells. LAMA-84 (A), U937 (B), HL-60 (C), or Jurkat (D) leukemia cells were exposed for 48 h to 200 nM STI in the presence or absence of equitoxic concentrations of FP, after which the percentage of apoptotic cells was determined as described in "Materials and Methods." FP concentrations were 150 nM (K562), 100 nM (HL-60), 200 nM (HL-60), and 75 nM (Jurkat). Values represent the means for three separate experiments performed in triplicate \pm SD. FP, flavopiridol; STI, STI571.

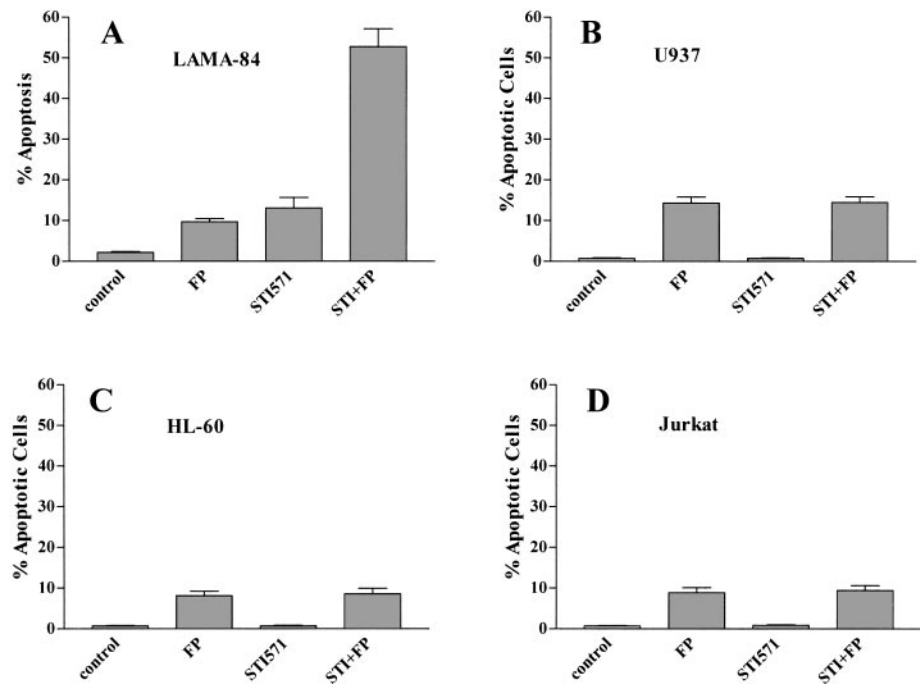


Table 1 Cell cycle analysis of K562 cells exposed to flavopiridol and STI571

Logarithmically growing K562 cells were exposed to 150 nM flavopiridol (FP) \pm 200 nM STI571 (STI) for 18 h, after which the percentage of cells in the G₀-G₁, S, and G₂-M phases of the cell cycle were determined by flow cytometry as described in "Materials and Methods." Values represent the means for three separate determinations \pm SD.

| | % cells | | | |
|--------------------------------|----------------|----------------|----------------|-----------------------------|
| | Control | FP | STI | STI + FP |
| G ₀ -G ₁ | 31.6 \pm 2.6 | 48.3 \pm 2.5 | 34.0 \pm 2.0 | 46.1 \pm 2.2 ^a |
| S | 48.3 \pm 3.1 | 37.2 \pm 3.1 | 50.4 \pm 2.1 | 33.1 \pm 3.0 ^a |
| G ₂ -M | 17.1 \pm 2.1 | 14.0 \pm 3.1 | 15.2 \pm 2.9 | 19.2 \pm 3.0 |

^a Not significantly different from values obtained for flavopiridol alone; $P > 0.05$.

particularly in the case of loss of $\Delta\psi_m$, denoting a highly synergistic interaction between these agents.

To gain insights into the hierarchy of apoptotic events occurring in response to STI571 + flavopiridol, K562 cells were treated with drugs in the presence of the pan-caspase inhibitor Boc-fmk (Fig. 2B). Coadministration of Boc-fmk largely blocked STI571/flavopiridol-mediated apoptosis and also diminished, although to a lesser extent, the loss of $\Delta\psi_m$. This finding indicates that STI571/flavopiridol-mediated disruption of the mitochondrial membrane potential represents, at least in part, a caspase-dependent phenomenon.

Effects of STI571 and flavopiridol on the cell cycle traverse of K562 cells are shown in Table 1. Exposure to 200 nM STI571 for 18 h had no effect on the cell cycle distribution of K562 cells, whereas 150 nM flavopiridol increased the G₀-G₁ population while producing a reciprocal decrease in the S-phase

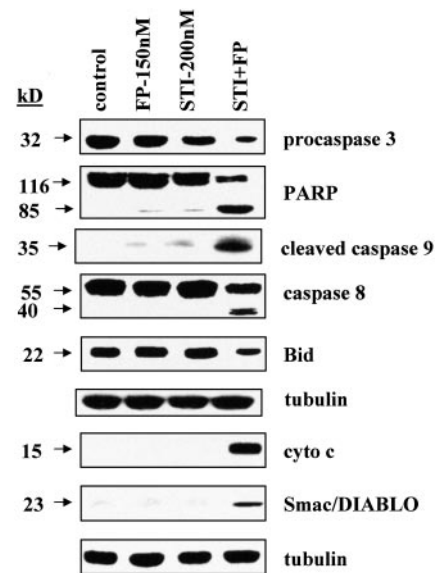


Fig. 4 Combined treatment of K562 cells with STI and FP markedly enhances procaspase-3, PARP, procaspase-8, procaspase-9, and Bid cleavage, and release of cyto *c* and Smac/DIABLO into the cytosolic S-100 fraction. K562 cells were exposed to 200 nM STI \pm 150 nM FP for 48 h, after which whole cells were lysed, proteins extracted, and subjected to Western analysis to monitor expression of full-length procaspase-3, PARP, a procaspase-9 cleavage fragment, procaspase-8, and Bid. Tubulin controls are shown to document equivalent loading and transfer of proteins. Alternatively, S-100 cytosolic fractions were obtained as described in "Materials and Methods" and monitored for expression of cyto *c* or Smac/DIABLO (second and third panels from the bottom). Separate tubulin controls are shown for the S-100 cytosolic fractions. In each case, lanes were loaded with 25 μ g of protein. The results of a representative experiment are shown; two other studies yielded equivalent results. FP, flavopiridol; STI, STI571; cyto *c*, cytochrome *c*.

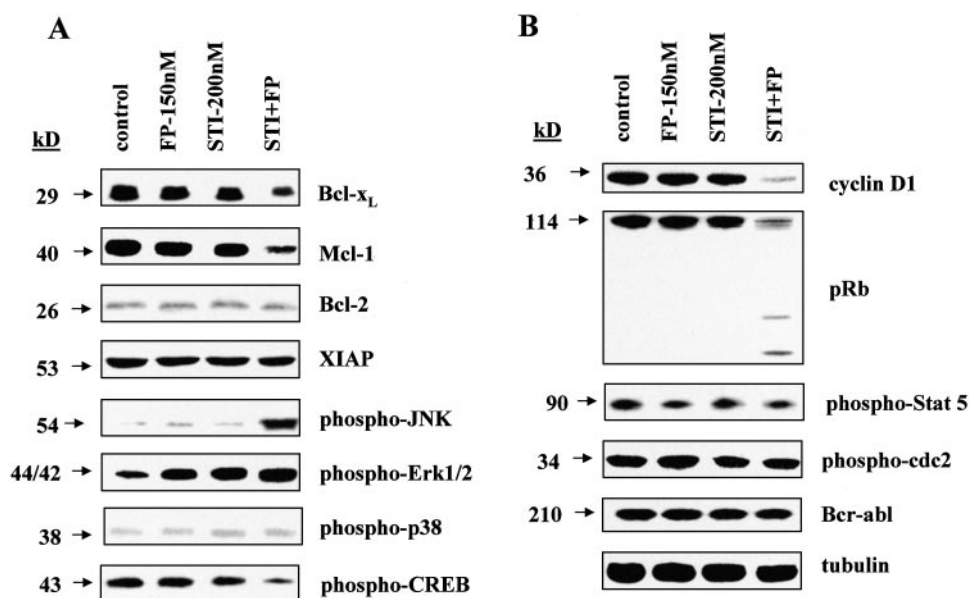


Fig. 5 Combined treatment of K562 cells with STI and FP induces down-regulation of Bcl-x_L, Mcl-1, pRb, cyclin D₁, and phospho-CREB and activation of JNK and ERK1/2 but does not modify Bcr/Abl protein expression. K562 cells were exposed to 200 nM STI ± 150 nM FP for 24 h, after which cells were lysed, proteins extracted, and subjected to Western analysis to monitor expression of Bcl-x_L, Mcl-1, Bcl-2, XIAP, phospho-JNK, phospho-ERK1/2, phospho-p38 MAPK, phospho-CREB (A), cyclin D₁, pRb, phospho-Stat5, phospho-p34^{cdc2}, and Bcr/Abl (B). Each lane was loaded with 25 μg of protein; blots were subsequently stripped and reprobed for expression of tubulin to ensure equivalent loading and transfer. The results of a representative experiment are shown; two other studies yielded equivalent results. FP, flavopiridol; STI, STI571; BOC, Boc-fmk.

fraction. The cell cycle distribution of cells exposed to the combination of STI571 and flavopiridol was essentially identical to that of cells exposed to flavopiridol alone. Values for the 24-h interval did not differ significantly from those obtained at 18 h (data not shown). These findings indicate that STI571, at least when administered at a concentration of 200 nM, does not modify flavopiridol-mediated G₀-G₁ arrest in K562 cells.

Combined treatment (48 h) with 150 nM flavopiridol and 200 nM STI571, which were minimally toxic when administered alone, resulted in a clear increase in apoptosis in LAMA 84 cells, another Bcr/Abl⁺ cell line (Fig. 3). In contrast, no potentiation of apoptosis was observed when equitoxic concentrations of flavopiridol were combined with STI571 in the three Bcr/Abl⁻ leukemic cell lines (U937, HL-60, and Jurkat; Fig. 4B–D), indicating that synergistic induction of cell death by STI571 and flavopiridol is restricted to leukemia cells expressing the Bcr/Abl tyrosine kinase.

Consistent with the marked increase in morphological evidence of apoptosis, 48-h coexposure of K562 cells to 200 nM STI571 + 150 nM flavopiridol resulted in a pronounced increase in cleavage/activation of procaspase-3, procaspase-8, procaspase-9, and Bid, as well as PARP degradation (Fig. 4). Combined treatment also resulted in a substantial increase in release of the proapoptotic mitochondrial proteins cytochrome *c*, which promotes caspase-9 activation, and Smac/DIABLO, which enhances caspase recruitment by opposing the actions of inhibitor of apoptosis proteins (25). Increases in cytosolic release of cytochrome *c* and Smac/DIABLO were also discernible at 24 h in STI571/flavopiridol-treated cells but were less pronounced than those observed at 48 h (data not shown). Together,

these findings indicate that combined exposure of K562 cells to STI571 and flavopiridol results in release of proapoptotic mitochondrial proteins accompanied by activation of the apoptotic caspase cascade.

Attempts were subsequently undertaken to assess the effects of STI571/flavopiridol on various apoptotic regulatory and signaling proteins. After exposure to 200 nM STI571 and 150 nM flavopiridol for 24 h and before the major onset of apoptosis (*i.e.*, Fig. 1, C and D), K562 cells exhibited a marked decline in levels of Bcl-x_L and Mcl-1 but no changes in levels of Bcl-2 or XIAP (Fig. 5A). Cells exposed to both flavopiridol and STI571 also displayed reduced expression of both pRb and cyclin D₁ (Fig. 5B). In the case of the former, rapidly migrating cleavage fragments were discernible. In addition, combined treatment resulted in a pronounced increase in phosphorylation of JNK and a decline in phosphorylation of CREB but no change in activation of p38 MAPK, p34^{cdc2}, or Stat5 (Fig. 5, A and B). Both STI571 and flavopiridol promoted ERK1/2 phosphorylation, and combined treatment resulted in an additional, although modest, increase in activation (Fig. 5A). The increase in ERK1/2 phosphorylation in STI571-treated K562 cells at this time interval is consistent with the results of our recent report (26). Notably, levels of Bcr/Abl protein were unperturbed after a 24-h exposure to STI571/flavopiridol (Fig. 5B).

To determine whether such changes might be secondary to caspase activation, cells were exposed to STI571 + flavopiridol in the presence of absence of Boc-fmk (Fig. 6). As anticipated, procaspase-3 cleavage was blocked by Boc-fmk. Similarly, Smac/DIABLO release was attenuated by administration of this caspase inhibitor, suggesting that this phenomenon is at least

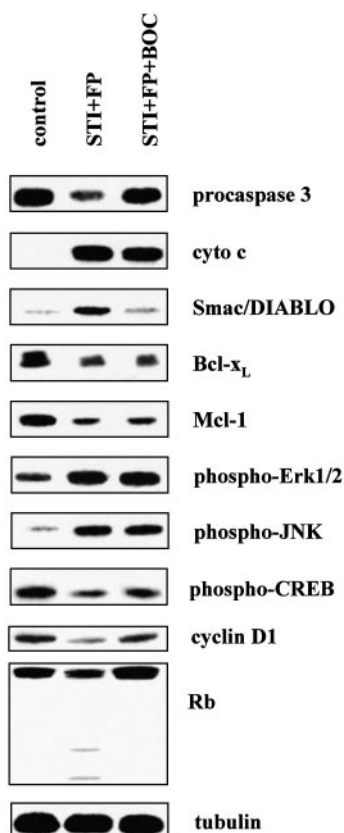


Fig. 6 STI571/FP-induced cyto *c* release, down-regulation of Bcl- x_L and Mcl-1, JNK/ERK1/2 activation are caspase-independent, whereas procaspase-3 cleavage, Smac/DIABLO release, diminished expression of pRb and cyclin D₁ and, to a lesser extent, down-regulation of phospho-CREB are caspase dependent. K562 cells were exposed to 200 nM STI + 150 nM FP in the presence or absence of 25 μ M BOC, after which cells were lysed, proteins extracted, and subjected to Western analysis to monitor expression of full-length procaspase-3 (48-h drug exposure), Bcl- x_L , Mcl-1, cyclin D₁, pRb, and phospho-JNK, phospho-ERK1/2, and phospho-CREB (24-h drug exposure in each case). Alternatively, S-100 cytosolic fractions were obtained after 48 h as described in Methods, and monitored for expression of cytochrome *c* or Smac/DIABLO. Each lane was loaded with 25 μ g of protein; blots were subsequently stripped and reprobed for expression of tubulin to ensure equivalent loading and transfer. The results of a representative experiment are shown; an additional study yielded equivalent results. cyto *c*, cytochrome *c*; FP, flavopiridol; STI, STI571.

partly caspase-dependent. Furthermore, the down-regulation of cyclin D₁ expression and pRb cleavage in STI571/flavopiridol-treated cells was largely prevented by Boc-fmk. In contrast to these events and unlike the loss of $\Delta\psi_m$ (Fig. 2B), cytochrome *c* redistribution was unperturbed by Boc-fmk, indicating that STI571/flavopiridol-mediated cytochrome *c* release proceeds independently of caspase activation. The reductions in levels of Bcl- x_L and Mcl-1 were also unaffected by caspase inhibition, indicating that these also represent caspase-independent events. Similarly, administration of Boc-fmk failed to modify STI571/flavopiridol-mediated potentiation of ERK1/2 and JNK activation. However, the decline in levels of phospho-CREB in STI571/flavopiridol-treated cells was slightly attenuated by caspase inhibition.

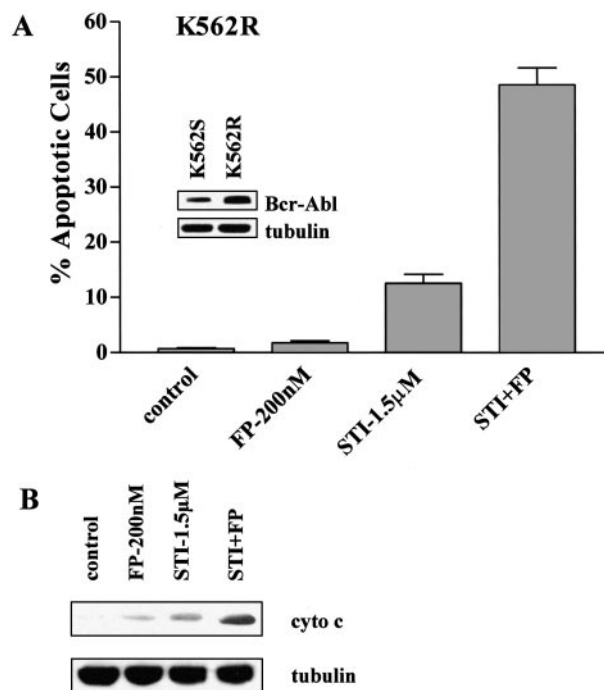


Fig. 7 Coexposure of drug-resistant K562 cells that express high levels of the Bcr/Abl protein to STI571 and flavopiridol results in a pronounced increase in apoptosis. **A**, K562R cells were exposed to 1.5 μ M STI \pm 200 nM FP for 48 h, after which the percentage of apoptotic cells was determined as described in "Materials and Methods." Relative levels of Bcr/Abl protein expression in parental (K562S) and K562R cells are shown in the accompanying Western blot (*inset*); each lane was loaded with 25 μ g of protein. Values represent the means for three separate experiments performed in triplicate \pm SD. **B**, K562R cells were exposed to the indicated concentrations of STI571 and flavopiridol for 48 h, after which S-100 cytosolic fractions were isolated as described in "Materials and Methods" and monitored for cyto *c* release by Western analysis. Each lane contained 25 μ g of protein; blots were stripped and reprobed with antibodies to tubulin to ensure equivalent loading and transfer. An additional experiment yielded equivalent results. cyto *c*, cytochrome *c*; FP, flavopiridol; STI, STI571.

To assess flavopiridol/STI571 interactions in cells otherwise resistant to STI571, a previously described (22) multidrug-resistant K562 cell line (K562R) was used, which we have very recently reported (26) exhibits approximately a 300% increase in Bcr/Abl protein expression relative to the parental line (Fig. 7A, *inset*). K562R cells display a marked decrease in STI571 susceptibility, with an IC₅₀ 10-fold higher than that of parental cells (e.g., \sim 3.0 *versus* 0.4 μ M in parental K562S cells; data not shown). In K562R cells, a 48-h exposure to 150 nM flavopiridol was nontoxic, whereas 1.5 μ M STI571 induced apoptosis in only \sim 10% of cells (Fig. 7A). In contrast, exposure of parental K562S cells to 1.5 μ M STI571 for 48 h resulted in $>$ 95% apoptosis (data not shown). Significantly, combined treatment of K562R cells with STI571 and flavopiridol increased the extent of apoptosis \sim 5-fold compared with STI571 alone (*i.e.*, to \sim 50%). Consistent with these results, treatment of K562R cells with the combination of STI571 and flavopiridol resulted in a marked increase in cytosolic release of cytochrome *c* (Fig. 7B). In separate studies, coadministration of the Pgp reversal

agent verapamil failed to increase the sensitivity of K562R cells to STI571 (data not shown), consistent with the results of our recent report (26). These findings indicate that coadministration of a subtoxic concentration of flavopiridol substantially potentiates STI571-related mitochondrial injury and lethality in resistant cells that exhibit increased levels of the Bcr/Abl protein.

DISCUSSION

The present results indicate that the CDK inhibitor flavopiridol markedly increases STI571-mediated mitochondrial damage and apoptosis in Bcr/Abl⁺ leukemic cells, including resistant cells that overexpress the Bcr/Abl protein. Over the last several years, various groups have demonstrated that combination of STI571 with conventional cytotoxic agents leads to enhanced killing of Bcr/Abl⁺ leukemia cells (27–29). In addition, potential synergism between STI571 and novel agents such as arsenic trioxide (30) and geldanamycin (31) has been reported. Very recently, our group has reported that pharmacological MEK1/2 inhibitors, including U0126, PD98059, and PD184352, interact in a highly synergistic manner with STI571 to induce cell death in Bcr/Abl⁺ cells, including resistant cells expressing high levels of the Bcr/Abl protein (26). Collectively, such findings raise the possibility that interruption of the Bcr/Abl signaling pathway, as well as one or more of its downstream targets, may render Bcr/Abl⁺ leukemic cells particularly susceptible to disruption of other survival/cell cycle regulatory cascades.

Although the events directly responsible for flavopiridol-mediated potentiation of STI571-related lethality remain to be determined, multiple possibilities exist. For example, flavopiridol has been shown to induce down-regulation of certain antiapoptotic proteins (*e.g.*, Bcl-2, XIAP; Ref. 32) in human leukemia cells, and up-regulation of Bcl-x_L has been implicated in the prosurvival activity of Bcr/Abl (6). Thus, the reduction in Bcl-x_L and Mcl-1 protein expression in K562 cells exposed to both flavopiridol and STI571 could plausibly have played a role in potentiation of apoptosis. Whether these events involve the recently described capacity of flavopiridol to act as an inhibitor of CDK9-mediated transcription (33) remains to be determined. In addition, the pronounced activation of JNK in STI571/flavopiridol-treated cells might also have contributed to enhanced lethality, a process that may involve posttranslational modifications in antiapoptotic proteins such as Bcl-2 (34). Analogously, diminished levels of phospho-CREB in cells exposed to STI571 and flavopiridol would also be expected to promote apoptosis (35). On the other hand, the observed activation of MAP kinase in STI571/flavopiridol-treated cells, an event generally associated with cytoprotective functions (36), in all likelihood represents a compensatory response to STI571/flavopiridol treatment, as recently described in the case of cells exposed to STI571 alone (26). In this context, the finding that MEK1/2 inhibitors, unlike flavopiridol, block rather than enhance MAP kinase activation in STI571-treated cells (26), suggests that such agents potentiate STI571 lethality through separate mechanisms. Finally, it is noteworthy that in contrast to cells exposed to STI571 and either arsenic trioxide (29), MEK1/2 inhibitors (26), or geldanamycin (30), cells treated with STI571 and flavopiridol did not display a decline in Bcr/Abl protein expression. This

finding suggests that combined treatment with the latter agents may act by disrupting cytoprotective pathways downstream of Bcr/Abl rather than by directly diminishing Bcr/Abl levels.

The possibility also exists that interactions between flavopiridol and STI571 might involve dysregulation of various cell cycle-related proteins. For example, flavopiridol-mediated apoptosis in breast cancer cells has been associated with down-regulation of cyclin D (17). Moreover, Bcr/Abl has been linked to increased expression of cyclin D₂ in hematopoietic cells (37). Interestingly, synergistic interactions between flavopiridol and the erbB2 antagonist Herceptin in human breast cancer cells have very recently been related to effects on cyclin D₁ (38). Thus, the possibility that enhanced apoptosis in flavopiridol/STI571-treated Bcr/Abl⁺ cells reflects perturbations in cyclin D expression/activity appears plausible. Indeed, combined exposure of K562 cells to flavopiridol and STI571 did lead to down-regulation of cyclin D₁, as well as diminished expression of pRb. However, the ability of the pan-caspase inhibitor Boc-fmk to block cyclin D₁ and pRb down-regulation suggests that these events represent consequences, rather than causes, of caspase activation. Nevertheless, the possibility that perturbations in such cell cycle regulatory proteins serve to amplify the apoptotic response to flavopiridol and STI571 cannot be excluded.

In addition to interfering with signaling pathways, flavopiridol may interact synergistically with STI571 by directly promoting mitochondrial damage. In this regard, whereas initial evidence suggested that flavopiridol initiated apoptosis independently of the mitochondrial pathway, at least in epithelial tumor cells (39), more recent findings indicate that in human leukemia cells, flavopiridol is a potent inducer of cytochrome *c* release (40). Consequently, the possibility that interruption of survival signaling downstream of Bcr/Abl (*i.e.*, by STI571) lowers the threshold for flavopiridol-induced mitochondrial injury seems plausible. It is noteworthy that in contrast to STI571/flavopiridol-mediated cytochrome *c* release, which was insensitive to caspase inhibition, Boc-fmk effectively blocked release of Smac/DIABLO, indicating that the latter event represents a secondary phenomenon. Such findings are concordant with recent evidence that in hematopoietic cells, mitochondrial release of cytochrome *c* and Smac/DIABLO proceed via separate pathways (41).

The finding that cotreatment with flavopiridol and STI571 resulted in a pronounced increase in apoptosis in STI571-resistant K562 cells that express increased levels of the Bcr/Abl protein is noteworthy. Although these cells were initially thought to represent a classic multidrug resistant line (22), the recent discovery (26) that they overexpress Bcr/Abl, which has been shown to confer leukemic cell resistance to multiple conventional cytotoxic agents (42), is likely to be pertinent. In this regard, the degree of Bcr/Abl overexpression in K562R cells is comparable with that of other STI571-resistant cell lines that have been described previously (42–44). Resistance of Bcr/Abl⁺ cells to STI571 may involve multiple mechanisms, including amplification of the Bcr/Abl protein, mutations in the Bcr/Abl kinase, pharmacokinetic factors, and diminished drug uptake, among others (14, 43–47). For reasons that are unclear, development of STI571 resistance in cells exposed to this agent *in vitro* is most commonly associated with increased expression

of the Bcr/Abl protein (44). Moreover, Bcr/Abl amplification has been reported in CML cells obtained from at least some patients who have become refractory to STI571 (14). It remains to be determined whether, and to what extent, coadministration of flavopiridol can overcome resistance stemming from other mechanisms, particularly Bcr/Abl mutations that may have particular clinical relevance (14).

In summary, the present findings demonstrate that coexpression of Bcr/Abl⁺ leukemia cells to the Bcr/Abl tyrosine kinase inhibitor STI571 in conjunction with the CDK inhibitor flavopiridol represents a potent stimulus for mitochondrial damage (e.g., cytochrome *c* and Smac/DIABLO release), caspase activation, and apoptosis. Moreover, this strategy is effective in at least some resistant cells that display increased levels of the Bcr/Abl protein. In addition, demonstration of synergism between STI571 and flavopiridol, particularly when these agents are administered at pharmacologically achievable concentrations (13, 16), may have translational implications. For example, binding of STI571 to serum proteins such as α_1 acidic glycoprotein (46) may reduce the effective concentration of free drug. However, coadministration of flavopiridol could potentially restore the ability of lower, free STI571 concentrations to kill Bcr/Abl⁺ cells. In the future, it will be important to identify the downstream Bcr/Abl pathways that may be directly targeted by flavopiridol and to determine whether this strategy is effective against cells resistant to STI571 through other clinically relevant mechanisms. Accordingly, plans to address these issues are currently under development.

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