The Cyclin-dependent Kinase Inhibitor Flavopiridol Disrupts Sodium Butyrate-induced p21$^{WAF1/CIP1}$ Expression and Maturation while Reciprocally Potentiating Apoptosis in Human Leukemia Cells[1]

Roberto R. Rosato, Jorge A. Almenara, Leanne Cartee, Vicki Betts, Srikumar P. Chellappan, and Steven Grant[2]

Department of Medicine, Medical College of Virginia, Virginia Commonwealth University, Richmond Virginia, 23298 [R. R. R., J. A. A., L. C., S. G.], and the Department of Pathology, Columbia University College of Physicians and Surgeons, New York, New York 10021 [V. B., S. P. C.]

Abstract

Interactions between the cyclin-dependent kinase inhibitor flavopiridol (FP) and the histone deacetylase inhibitor sodium butyrate (SB) have been examined in human leukemia cells (U937) in relation to differentiation and apoptosis. Whereas 1 mM of SB or 100 nM of FP minimally induced apoptosis (4% and 10%, respectively) at 24 h, simultaneous exposure of U937 cells to these agents dramatically increased cell death (*e.g.,* ~60%), reflected by both morphological and Annexin/propidium iodide-staining features, procaspase 3 activation, and poly(ADP-ribose) polymerase cleavage. Similar interactions were observed in human promyelocytic (HL-60), B-lymphoblastic (Raji), and T-lymphoblastic (Jurkat) leukemia cells. Coadministration of FP opposed SB-mediated accumulation of cells in G_0G_1 and differentiation, reflected by reduced CD11b expression, but instead dramatically increased procaspase-3, procaspase-8, Bid, and poly(ADP-ribose) polymerase cleavage, as well as mitochondrial damage (*e.g.,* loss of mitochondrial membrane potential and cytochrome *c* release). FP also blocked SB-related p21$^{WAF1-CIP1}$ induction through a caspase-independent mechanism and triggered the caspase-mediated cleavage of p27^{KIP1} and retinoblastoma protein. The latter event was accompanied by a marked reduction in retinoblastoma protein/E2F1 complex formation. However, FP did not modify the extent of SB-associated acetylation of histones H3 and H4. Treatment of cells with FP/SB also resulted in the caspase-mediated cleavage of Bcl-2 and caspase-independent down-regulation of Mcl-1. Levels of cyclins A, D_1, and E, and X-linked inhibitor of apoptosis also declined in SB/FP-treated cells. Finally, FP/SB coexposure potently induced apoptosis in two primary acute myelogenous leukemia samples. Together, these findings demonstrate that FP, when combined with SB, induces multiple perturbations in cell cycle and apoptosis regulatory proteins, which oppose leukemic cell differentiation but instead promote mitochondrial damage and apoptosis.

Introduction

SB[3] is a nontoxic short-chain fatty acid found naturally in the gastrointestinal tract that appears to be responsible for the protective effects attributed to high-fiber diets (1). In this regard, SB has been shown to exhibit activity in a rat model of colon cancer (2), to suppress proliferation of cancer cell lines (3), and to increase markers of cell differentiation (4). Like other HDIs, it promotes acetylation of histones, leading to alterations in chromosomal structure that promote expression of genes involved in cellular maturation (5). It is also able to induce tumor cell apoptosis in a dose-dependent manner (3). From a functional standpoint, SB increases the expression of the cdki p21$^{WAF1/CIP1}$, which leads in turn to growth arrest in the G_1 phase of the cell cycle (6). In contrast to what has been observed in primary cultures of fibroblasts obtained from transgenic p21$^{WAF1/CIP1}$ $-/-$ mice (6), it has been shown that p21$^{WAF1/CIP1/MDA6}$ plays an important role in promoting differentiation and preventing mitochondrial dysfunction and apoptosis in human leukemia cells (*i.e.,* HL-60 and U937; Refs. 7, 8). For example, U937 cells stably transfected with a p21$^{WAF1/CIP1}$ antisense construct were significantly more susceptible to 1-β-D-arabinofuranosylcytosine- and SB-mediated mitochondrial injury and apoptosis than their wild-type counterparts (4, 9). Similar findings have been reported in cells exposed to vitamin D_3 (10). Together, these findings suggest that leukemic cells displaying dysregulation of this cdki respond to SB and other differentiation inducers by engaging a default apoptotic program.

p21$^{WAF1/CIP1}$ is a member of a family of cdkis, which also includes p27^{KIP1} and p57 (11). It inhibits multiple cdks, in-

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[2] To whom requests for reprints should be addressed, at Medical College of Virginia, Virginia Commonwealth University, MCV Station Box 230, Richmond, VA 23298. Phone: (804) 828-5211; Fax: (804) 828-8079; E-mail: stgrant@hsc.vcu.edu.

[3] The abbreviations used are: SB, sodium butyrate; cdki, cyclin-dependent kinase inhibitor; cdk, cyclin-dependent kinase; FP, flavopiridol; PKC, protein kinase C; AML, acute myelogenous leukemia; HDI, histone deacetylase inhibitor; PMA, phorbol 12-myristate 13-acetate; $\Delta\psi_m$, mitochondrial membrane potential; PI, propidium iodide; PBS-T, PBS-Tween; $DiOC_6$, 3,3-dihexlyoxacarbocyanine; XIAP, X-linked inhibitor of apoptosis; Boc-D-fmk, BOC-Asp (OMe)-fluoromethyl ketone; PARP, poly(ADP-ribose) polymerase; EMSA, electrophoretic mobility shift assay; IP, immunoprecipitation; pRb, retinoblastoma protein.

cluding cdk1, cdk2, cdk4, and cdk6 (12). $p21^{WAF1/CIP1}$ comprises part of a multiprotein complex which includes cyclins, cdks, and the proliferating nuclear cell antigen (13). When induced in response to various stimuli, $p21^{WAF1/CIP1}$ expression results in the inhibition of G_1 cyclin/cdk complexes and G_1 arrest (14). This effect has been related, at least in part, to dephosphorylation of the pRb, which then binds to and inactivates the transcription factor E2F (15). In some settings, $p21^{WAF1/CIP1}$ promotes differentiation (16). However, in many tissues, induction of $p21^{WAF1/CIP1}$ correlates with the initiation of but not necessarily the establishment of a terminally differentiated phenotype (17). Both p53-dependent and -independent pathways of $p21^{WAF1/CIP1}$ induction in cells exposed to DNA-damaging and maturation/differentiation-inducing agents have been described (17–20).

FP is a pharmacological cdki that interacts with the adenine-binding pocket of cdks, resulting in a marked inhibition of kinase activity (21). It is the first cdk modulator to enter clinical trials in humans (22). FP has also been reported to decrease the expression of cyclins D_1, D_3, and E without modifying cyclin D_2 protein levels (23). FP induces either G_1 and/or G_2M cell cycle arrest concordantly with its ability to block the expression of various cyclins and inhibit cdk activity. Furthermore, FP is a potent inducer of apoptosis in human leukemia cells (24) and increases the lethality of conventional cytotoxic agents in a sequence-dependent manner (25). Although the mechanism by which FP promotes apoptosis in leukemia cells remains unknown, it is presumed that FP-mediated cytotoxicity stems from cell cycle perturbations, particularly in view of abundant evidence that disruption of cell cycle progression represents a potent apoptotic stimulus (26). FP has also been reported to induce maturation of non-small lung cancer cells (27), a capacity that may reflect the critical role that cell cycle arrest (*i.e.,* in G_1) plays in the differentiation process (28).

We reported previously that coadministration of FP with the tumor-promoter and PKC activator PMA blocked expression and reporter activity of $p21^{WAF1/CIP1}$ in human leukemia cells (U937), events associated with induction of apoptosis (29). In light of evidence that $p21^{WAF1/CIP1}$ plays a critical role in differentiation/apoptosis induction by SB (4) and other HDIs (30), the possibility that FP could exert similar effects in SB-treated leukemic cells appeared plausible. Currently, no information exists regarding interactions between FP and HDIs such as SB, either in hematopoietic or nonhematopoietic neoplastic cells. The goal of the present study was to determine whether cell cycle arrest induced by FP would promote SB-mediated maturation in human leukemia cells or, alternatively, result in potentiation of cell death. Our results indicate that exposure of human leukemia cells to FP blocks SB-mediated $p21^{WAF1/CIP1}$ induction, G_1 arrest, and maturation. Instead, FP promotes SB-related mitochondrial damage (*i.e.,* cytochrome *c* release and loss of $\Delta\Psi_m$) activation of procaspases-3, 8, and Bid, degradation of PARP, pRb, $p27^{KIP1}$, and Bcl-2, diminished pRb/E2F-1 complex formation, and down-regulation of Mcl-1, XIAP, and cyclins D1, A, and E. Significantly, qualitatively similar interactions were observed between SB and FP in two primary AML specimens. Together, these findings indicate that exposure of SB-treated leukemic cells to FP leads to interference with induction of $p21^{WAF1/CIP1}$ and a variety of other perturbations in cell cycle and apoptosis regulatory events that culminate in mitochondrial damage and apoptosis.

Materials and Methods

Cells. The myelomonocytic leukemia cell line U937 was derived from a patient with histiocytic lymphoma and was obtained from American Type Culture Collection (Rockville, MD). HL-60 promyelocytic leukemia cells, Raji B-lymphoblastic leukemia cells, and Jurkat T-lymphoblastic leukemia cells were also purchased from American Type Culture Collection. Cells were cultured and maintained in logarithmic growth phase in RPMI 1640 supplemented with sodium pyruvate, MEM essential vitamins, L-glutamate, penicillin, streptomycin, and 10% fetal bovine serum (Life Technologies, Inc.). Cells were maintained in a 37°C, 5% CO_2, fully humidified incubator and passed twice weekly.

Drugs and Reagents. SB was supplied as a powder (Calbiochem, La Jolla, CA) and dissolved in PBS before use. FP (L86 8275; NCS 649890) was kindly provided by Dr. Edward Sausville (Cancer Treatment and Evaluation Program, National Cancer Institute, Bethesda, MD). FP was formulated in DMSO, and 10^{-2} M stock solutions were stored at −20°C.

Antibodies for Western Analysis. Primary antibodies for both $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (Transduction Laboratories, Lexington, KY) were used in a 1:1000 dilution; PARP, 1:1000 (Oncogene Research Products, Cambridge, MA); procaspase 3, 1:1000 (Transduction Laboratories); cytochrome *c*, 1:2000 (PharMingen, San Diego, CA); bcl-2, 1:2000 (DAKO, Glostrup, Denmark); bax, 1:1000 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); bid, 1:1000 (Cell Signaling, Beverly, MA); XIAP, 1:1000 (Cell Signaling); Mcl-1, 1:1000 (PharMingen); bak, 1:1000 (Calbiochem, San Diego, CA); c-Myc, 1: 1000 (PharMingen); actin, 1:2000 (Sigma Chemical Co. Chemicals, St. Louis, MO); cyclins-D1, -A, and -E, 1:1000 (PharMingen); pRb, underP-pRb, pRb-cdk2, and pRb-cdk4, all 1:1000 (PharMingen); Ac-H3 and Ac-H4, both 1:1000 (Upstate Biotechnology, Lake Placid, NY); and E2F-1 (Upstate Biotechnology). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Coomassie protein assay reagent was purchased from Pierce (Rockford, IL), and an enhanced chemiluminescence kit was obtained from New England Nuclear (Boston, MA). The pan-caspase inhibitor BOC-D-fmk and the caspase 8 inhibitor Z-IETD-fmk were purchased from Enzyme System Products (Livermore, CA). All of the other chemicals or reagents were purchased from Sigma Chemical Co.

Assessment of Apoptosis. Apoptotic cells were evaluated by either morphological assessment of Wright-Giemsa-stained cytospin preparations or Annexin V/PI analysis. Morphologically, apoptotic cells were identified by classical features (*i.e.,* nuclear condensation, cell shrinkage, and formation of apoptotic bodies). Five or more randomly selected fields, encompassing a total of ≥1000 cells/slide, were evaluated to determine the percentage of apoptotic cells for each treatment condition as described previously (7). Alternatively, Annexin V/PI assay was carried out as per the manufacturer's instruc-

tions (BD PharMingen, San Diego, CA) using a Becton Dickinson FACScan cytofluorometer (Mansfield, MA).

Cell Cycle Analysis. After drug treatment, cells were pelleted by centrifugation at 500 $g \times 6$ min and resuspended in 70% ethanol. The cells were incubated on ice for $\geq$1 h and resuspended in 1 ml of cell cycle buffer (0.38 mM of Na-citrate, 0.5 mg/ml RNase A, and 0.01 mg/ml PI) at a concentration of 10×10^5 cells/ml, stored in the dark at 4°C until analysis (24 h), using a Becton Dickinson FACScan flow cytometer and Verity Winlist software (Verity Software, Topsham, ME).

Assessment of $\Delta\psi_m$. At the indicated intervals, cells were harvested, and 2×10^5 cells were incubated with 40 nM of $DiOC_6$ for 15 min at 37°C in PBS. Analysis was then carried out on Becton Dickinson FACScan cytofluorometer. The percentage of cells exhibiting low levels of $DiOC_6$, reflecting loss of $\Delta\psi_m$, was determined.

Western Analysis. Whole cell pellets were washed twice in PBS, resuspended in PBS, and lysed by the addition of 1 volume of loading buffer [2× loading buffer (60 mM Tris base, pH 6.8, 4% of SDS, 5.76 mM β-mercapto ethanol, 20% glycerol, 0.2% bromo phenol blue)]. Lysates were boiled for 10 min, centrifuged at 12,800 $\times$ g for 5 min, and quantified using Coomassie protein assay reagent (Pierce). Total proteins (25 μg/point) were separated by SDS-PAGE and electroblotted to nitrocellulose. The blots were blocked in 5% nonfat milk in PBS-T and probed for 1 h with the appropriate dilution of primary antibody. Blots were washed 3 $\times$ 10 min in PBS-T and then incubated with a 1:2000 dilution of peroxidase-conjugated secondary antibody for 1 h at room temperature. Blots were again washed 3 $\times$ 10 min in PBS-T and then developed by enhanced chemiluminescence. Where indicated, blots were stripped and reprobed with antibodies directed against actin.

Histone Deacetylase Assay. Acid extraction of proteins from treated cells and detection of acetylated histones H3 and H4 by Western blot analysis was performed per the manufacturer's instructions (Upstate Biotechnology). Extract (10 μg/lane) was loaded and separated on precast 4–20% Bio-Rad Gradient Gels.

Analysis of Cytosolic Cytochrome *C*. A technique described previously was used (9). After treatment, cells (5×10^7/condition) were harvested by centrifugation at 600 $\times$ g for 10 min at 4°C. The S-100, or cytosolic fraction, was prepared as described with minor modifications. Cell pellets were washed once with ice-cold PBS and resuspended in five volumes of buffer A [20 mM of HEPES (4-(2-hydro-ethyl)-piperazine-1-ethane-sulfonic acid)-KOH (pH 7.5), 10 mM of KCl, 1.5 mM of $MgCl_2$, 1 mM of sodium EDTA, 1 mM of sodium EGTA, 1 mM of DTT, 0.1 mM of phenylmethlysufonyl fluoride, and 250 mM of sucrose; all Sigma Chemical Co.]. After chilling for 30 min on ice, the cells were disrupted by 15 strokes of a Potter-Elvehjem (PTFE-pestle) homogenizer. The homogenate was then centrifuged twice to remove unbroken cells and nuclei (3000 $\times$ g for 10 min at 4°C). The resulting supernatant was additionally centrifuged at 100,000 $\times$ g for 1 h at 4°C, and the supernatants, designated as S-100 fractions, were immediately subjected to Western analysis as described above. For each condition, 25 μg of the S-100 fraction was loaded on the gel and probed with the corresponding antibody.

Differentiation Studies. Expression of the monocytic differentiation marker CD11b was monitored by direct immunofluorescence staining and flow cytometric analysis as described previously (7). After drug treatment, suspension and adherent cells were enumerated by a Coulter Counter, and 2×10^6 cells were pelleted by centrifugation. The supernatant was aspirated, and the cells were resuspended in 300 μl of ice-cold PBS. Two 100-μl aliquots from each sample were then combined with either phycoerythrin-1 (10 μl) or the IgG control. Samples were incubated for 20 min at 4°C and diluted in PBS (1 ml). Sample data were collected using a Becton Dickinson FACScan flow cytometer and analyzed with Verity Winlist Software (Verity Software).

IP and EMSA. Whole-cell extracts were prepared by hypotonic shock followed by salt extraction, as described previously (31). Portions (50–200 μg) of whole-cell extracts were treated with 5 μl of the appropriate primary antibody in a volume of 100 μl at 4°C for 1 h. Then, 3 mg of protein A-Sepharose or protein G-Sepharose in a 100-μl volume was added and incubated for an additional hour. The binding was performed in a buffer containing 20 mM of HEPES (pH 7.9), 40 mM of KCl, 1 mM of MgCl2, 0.1 mM of EGTA, 0.1 mM of EDTA, 0.1 mM of DTT, 0.1 mM of NaF, 0.1 mM of Na3VO4, 0.5% NP40, and 3 mg of BSA/ml. The beads were washed six times with 600 μl of the same buffer and boiled in 20 μl of SDS sample buffer. EMSA after IP was performed as described previously (31). An *Eco*RI-*Hin*dIII fragment of the adenovirus E2 promoter containing two E2F binding sites (TTTCGCGC) was end labeled by using Klenow fragment and was used as the probe in all of the assays. Briefly, 8 μg of whole-cell extracts were incubated with ~0.2 ng of ^{32}P-labeled E2F probe in a buffer containing 20 mM HEPES (pH 7.9), 40 mM KCl, 0.1 mM concentrations each of MgCl2, EGTA, EDTA, DTT, NaF, and Na3VO4; 1% NP40, 1 μg of salmon sperm DNA/ml, and 10 μg of BSA/ml. After incubation at room temperature for 20 min, the reactions were separated on a 4% polyacrylamide gel in 0.25 $\times$ Tris-borate EDTA at 300 V for 3 h. The gel was dried, and the bands were detected by autoradiography.

Isolation of Patient-derived Leukemic Blasts. Leukemic blasts were obtained with informed consent from the peripheral blood of patients with AML. These studies have been approved by the Investigational Review Board of the Medical College of Virginia/Virginia Commonwealth University. Blood was collected in sterile syringes containing heparin diluted 1:4 with cell culture medium (RPMI 1640) and aliquoted into 50-ml sterile plastic centrifuge tubes. Only samples consisting of >70% blasts, and which displayed >90% viability by trypan blue exclusion after isolation, were used in these studies. To each tube was added an underlayer of sterile Ficoll-Histopaque (sp grav.1.077; Sigma Chemical Co.-Aldrich, St. Louis, MO), and the tubes were centrifuged for 30 min at 400 $\times$ g at room temperature. Cells were then harvested from the interface layer, resuspended in fresh medium, and prepared for studies as above.

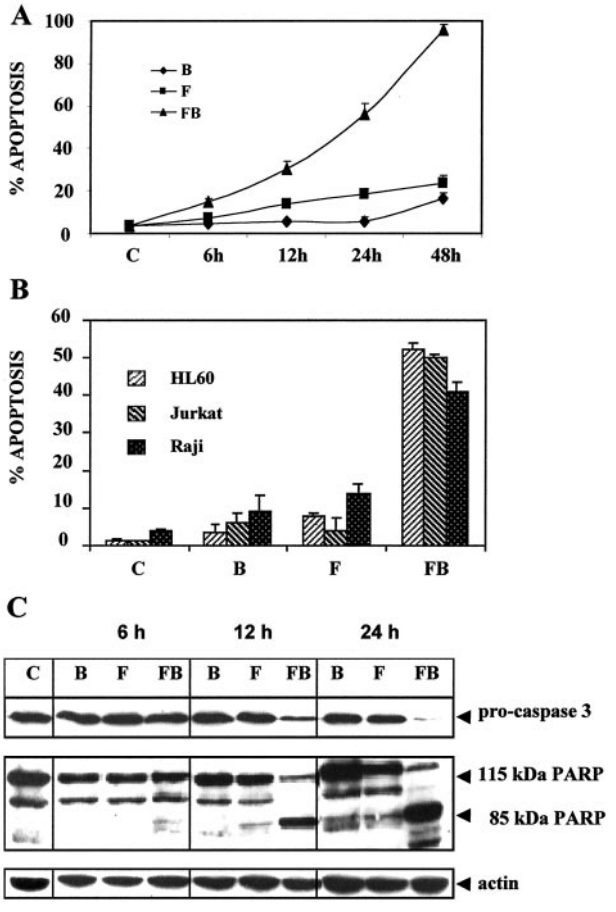

Fig. 1. In *A*, human monocytic leukemia cells (U937) were exposed to 1 mM SB (*designated B*), 100 nM FP (*designated F*), or the combination (*designated FB*) for 6, 12, 24, or 48 h, after which cytospin preparations were obtained, stained with Wright-Giemsa, and the percentage of apoptotic cells determined as described in "Materials and Methods." In *B,* the leukemic cell lines HL-60 promyelocytic, Jurkat T-lymphoblastic, and Raji B-lymphoblastic leukemia were exposed to 1 mM SB, 100 nM FP, or both agents for 24 h, after which the extent of apoptosis cells determined as described in the text. For *A* and *B*, values represent the means for three separate experiments performed in triplicate; *bars,* ± SD. *C*, Western blot analysis of caspase-3 and PARP cleavage. U937 cells were incubated for designated interval with 1 mM SB ± 100 nM FP after which they were pelleted, lysed, and 30 μg of protein separated by SDS-PAGE. Blots were then probed with antibodies directed against procaspase-3 or PARP, after which they were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results.

Table 1 Time course of cell death induction by FP ± SB in U937 cells as determined by annexin V/PI staining

U937 monocytic leukemia cells were exposed for the indicated intervals to 100 nM FP (F), 1 mM SB (B), or both agents in combination, stained with annexin V/PI, and cell death analyzed by flow cytometry as described in "Materials and Methods." Values, corresponding to the percentage of dead cells, represent the means ± SD for three separate experiments performed in triplicate.

	6 h	12 h	24 h	48 h
C	4.5 ± 1.2	4.3 ± 1.3	4.2 ± 0.8	4.7 ± 1.4
B	4.2 ± 0.8	4.9 ± 1.7	7.3 ± 1.3	14.6 ± 1.4
F	7.5 ± 1.3	12.5 ± 1.8	18.1 ± 2.3	24.3 ± 2.6
FB	15.5 ± 2.1	35.8 ± 7.1	52.5 ± 4.7	86.7 ± 5.6

Results

Cotreatment with FP and SB Results in a Marked Apoptotic Response in Multiple Human Leukemia Cell Lines. The effects of a combined exposure to FP and SB on apoptosis in U937 cells are shown in Fig. 1*A*. Whereas exposure to 100 nM FP or 1 mM SB alone for 12–24 h exerted minimal toxicity toward these cells (*e.g.,* 15% apoptosis), combined treatment resulted in a pronounced increase in morphological evidence of cell death (*e.g.,* to ~ 60% at 24 h). Moreover, a 48-h exposure to SB/FP induced apoptosis in virtually 100% of cells. Quantification of cell death by flow cytometric analysis of Annexin/PI-stained samples yielded essentially equivalent results (Table 1). To determine whether these findings could be extended to other leukemia cell lines, HL-60 promyelocytic, Jurkat T-lymphoblastic, and Raji B-lymphoblastic human leukemic cells were exposed for 24 h to 1 mM SB, 100 nM FP, or the combination of both agents, after which the apoptosis was monitored (Fig. 1*B*). As in the case of U937 cells, SB or FP administered alone were minimally toxic at 24 h, generally inducing apoptosis in ≤15% of cells. In marked contrast, simultaneous exposure of each of the cell lines to both agents in combination markedly increased apoptosis (*e.g.,* to 45–55% of cells. Together, these findings indicate that the marked induction of apoptosis by SB and FP is neither lineage- nor cell-type specific. Consistent with these results, activation of procaspase-3, manifested by cleavage/degradation of the full-length M_r 32,000 species, was considerably more pronounced in FP/SB-treated U937 cells at 12 and 24 h compared with the effects of either agent administered alone (Fig. 1*C*). Similarly, degradation of the caspase-3 substrate, PARP, from a M_r 115,000 full-length species to a M_r 85,000 cleavage product was observed in parallel (Fig. 1*C*). Dose-response studies (Table 2) revealed that 24-h exposure of U937 cells to an SB concentration of 0.5 mM, which was nontoxic by itself, substantially increased the lethal effects of FP concentrations ranging from 50 to 300 nM. The most dramatic increase in apoptosis was noted when 2 mM SB, which was minimally toxic at 24 h (*e.g.,* 6.1 ± 0.8% apoptotic), was combined with modestly toxic FP concentrations (*e.g.,* 100–150 nM). Such combinations induced apoptosis in 75–85% of cells after 24 h of drug exposure. These findings indicate that the cdki FP and the HDI SB potently trigger the cell death cascade in multiple human leukemia cell types.

FP/SB-mediated Apoptosis Primarily Involves the Intrinsic Apoptotic Pathway. Treatment of U937 cells with the pan-caspase inhibitor BOC-fmk blocked FP/SB-induced apoptosis after 12 h of exposure, whereas no inhibition was observed when the caspase-8 inhibitor IEDT-fmk was used (Fig. 2*A*). Procaspase-8 activation, reflected by the appearance of a cleavage fragment, became clearly evident as early as 6 h after treatment with FP/SB, an event followed by a decrease in levels of the caspase-8 substrate Bid (Fig. 2*B*). Cleavage of caspase-8 and Bid were blocked by both the caspase-8 inhibitor IEDT-fmk (Fig. 2*B*) or the pan-caspase inhibitor BOC-fmk (data not shown). Together, these findings suggest that activation of the extrinsic, caspase-8-depend-

Table 2 Induction of apoptosis after a 24-h exposure of U937 cells to varying concentrations of FP or SB, administered alone and in combination

U937 monocytic leukemia cells were exposed to the indicated concentrations of FP, SB, or both agents in combination for 24 h, after which the percentage of apoptotic cells was determined by morphological examination of Wright and Giemsa-stained cytospin preparations. Values represent the means ± SD for three separate experiments performed in triplicate.

	C	FP 50 nM	FP 75 nM	FP 100 nM	FP 150 nM	FP 300 nM
C	0.9 ± 0.4	6.1 ± 1.3	13.9 ± 0.5	14.5 ± 3.1	24.2 ± 0.5	61 ± 1.4
SB 0.5 mM	1.6 ± 0.6	13.8 ± 1.7	26.6 ± 4.2	26.6 ± 3.3	36.6 ± 3.5	91 ± 3.5
SB 1 mM	1.7 ± 0.5	12.3 ± 1.8	24.3 ± 3.2	55.4 ± 1.3	76.2 ± 1.4	94 ± 1.8
SB 2 mM	6.1 ± 0.8	40.2 ± 7.1	47.4 ± 0.9	75.5 ± 0.7	85.3 ± 2.5	95 ± 3.5

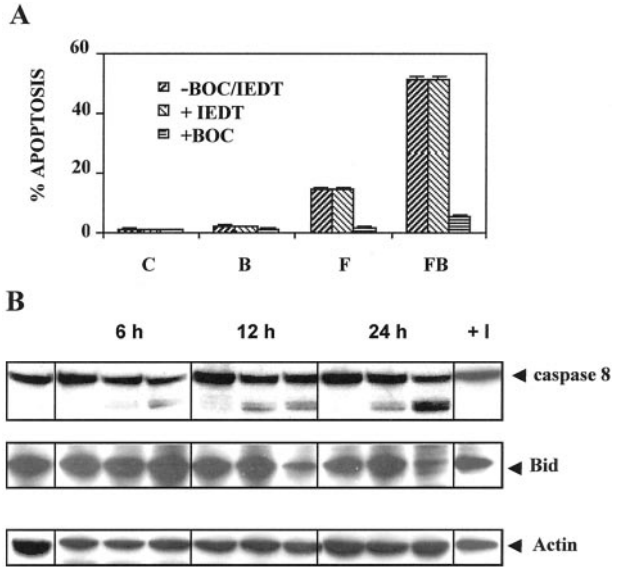

Fig. 2. *A*, U937 cells were treated for 12 h with 100 nM FP (*F*), 1 mM SB (*B*), or the combination in the absence or presence of either 20 μM of the pan-caspase inhibitor BOC-D-fmk or the caspase-8 inhibitor Z-IEDT-fmk. Apoptosis was determined by morphological analysis of Wright/Giemsa-stained cytospin preparations as described above; *bars,* ± SD. *B*, Western blot analysis of caspase-8 and its substrate Bid. After incubation with 100 nM FP ± 1 mM SB for 12 h, cells were pelleted, lysed, and 30 μg of protein separated by SDS-PAGE and blotted with the designated antibodies. Blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results.

ent pathway represents a secondary event in cells exposed to the combination of SB and FP.

Enhanced Apoptosis in U937 Cells Exposed to FP/SB Is Associated with a Primary Cytochrome *C* Release and a Secondary Loss of $\Delta\psi_m$. Early mitochondrial dysfunction has been shown to play important role in apoptotic events (32). To determine what effect exposure of cells to SB and FP might have on mitochondrial perturbations accompanying (or responsible for) apoptosis, cytoplasmic cytochrome *c* release and uptake of the lipophilic fluorochrome $DiOC_6$, reflecting maintenance of $\Delta\psi_m$, were monitored. Cells exposed to 100 nM FP alone displayed a modest increase in the percentage of cells displaying a loss of $\Delta\psi_m$ at 12 or 24 h (Fig. 3*A*), consistent with our previous findings (33). However, coexposure of cells to 1 mM SB, which by itself exerted no effect on $\Delta\psi_m$, resulted in a clear increase in mitochondrial discharge. Significantly, the loss of $\Delta\psi_m$ in SB/FP-treated cells was blocked by the pan-caspase inhibitor Boc-fmk (Fig. 3*A*). In contrast, cytochrome *c* release, which increased modestly as early as 1 h after treatment with SB or FP alone, became very pronounced in cells exposed to both drugs for intervals ≥6 h (Fig. 3*B*). Moreover, in contrast to the loss of $\Delta\psi_m$, SB/FP-mediated cytochrome *c* release was not inhibited by Boc-fmk. These findings indicate that combined treatment of U937 cells with SB and FP is associated with early potentiation of mitochondrial injury, in which cytochrome *c* release and $\Delta\psi_m$ loss represent primary and secondary events, respectively.

FP Opposes the Accumulation of Cells in G_0/G_1 and Disrupts Maturation in SB-treated U937 Cells. FP is a potent cdki (34) and induces arrest in various phases of the cell cycle, depending on the cell type (35). SB is also known to induce cell cycle arrest (4, 36). To determine what effect combined treatment with SB and FP would have on cell cycle traverse, the cell cycle distribution of U937 cells was examined 24 and 48 h after administration of 1 mM SB, 100 nM FP, or the combination (Fig. 4*A*). SB-treated cells exhibited a prominent increase in the percentage of cells arresting in G_0/G_1 (*e.g.,* 48.1% and 81.6% at 24 and 48 h, respectively). Arrest in G_0/G_1 after SB treatment was accompanied by increased expression of the differentiation marker CD11b (Fig. 4*B*). FP-treated cells also exhibited an increase in the G_0/G_1 population at the 48-h interval (*i.e.,* ~53%; Fig. 4*A*), although in contrast to SB, this was not accompanied by an increase in CD11b expression (Fig. 4*B*). However, when cells were treated with the combination of FP and SB, a large increase in the subdiploid (apoptotic) fraction was noted at 24 and particularly at 48 h, thus opposing the accumulation of cells in G_0/G_1 that was observed in cells exposed to SB (Fig. 4*A*). Furthermore, FP coadministration antagonized SB-induced differentiation manifested by a marked reduction the percentage of CD11b-expressing cells (Fig. 4*B*). These findings indicate that despite promoting cell cycle arrest, FP does not enhance SB-related G_0/G_1 accumulation and differentiation but instead causes cells to engage an alternative, apoptotic program.

Coexposure to FP Blocks SB-induced Expression of the cdki $p21^{WAF1/CIP1}$. Previous studies have demonstrated the importance of the cdki p21WAF1/CIP1 in leukemic cell maturation, including that induced by SB (4, 29, 37). Therefore, the effects of FP on SB-mediated induction of $p21^{WAF1/CIP1}$ were examined (Fig. 5*A*). Administration of 1 mM SB induced a time-dependent increase in levels of $p21^{WAF1/CIP1}$, which was first detectable at 1–3 h, and which was very robust at exposure intervals ≥12 h. FP alone did not modify $p21^{WAF1/CIP1}$ expres-

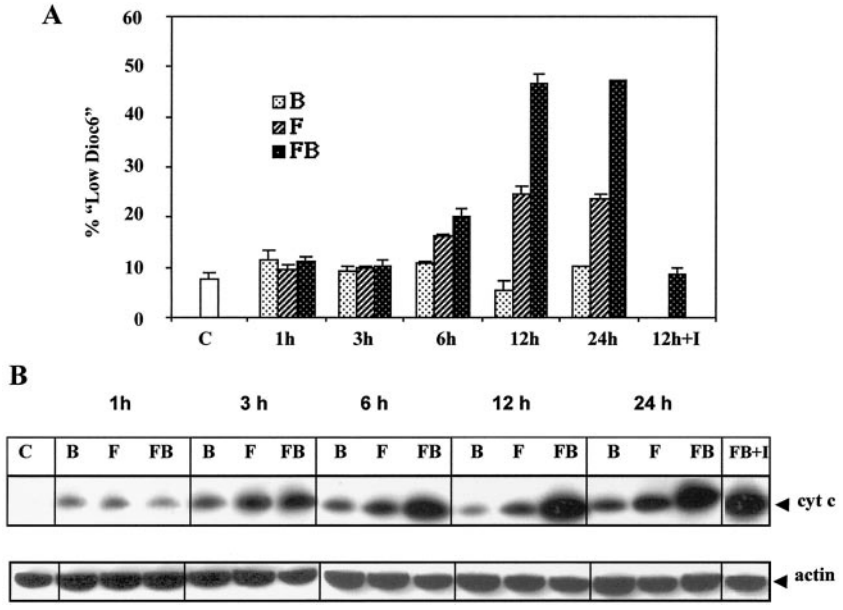

Fig. 3. *A*, The loss of $\Delta\psi_m$ was monitored at the indicated time points in U937 cells exposed to 100 nM FP (*F*)/1 mM SB (*B*). Values represent the means for three separate experiments performed in triplicate and are expressed as the percentage of cells expressing low $\Delta\psi_m$, reflected by reduced levels of $DioC_6$ uptake, relative to untreated controls; *bars,* ± SD. *B*, Western blot analysis of the time course release of cytochrome *c* into the cytosolic S-100 fraction in U937 cells exposed to SB and FP as above. Blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. Results from a representative study are shown; similar results were obtained in two additional experiments.

sion. However, coadministration of FP essentially abrogated induction of $p21^{WAF1/CIP1}$ by SB at each interval examined. Significantly, this effect was not prevented by the pan-caspase inhibitor Boc-D-fmk (20 μM), indicating that FP interferes with $p21^{WAF1/CIP1}$ induction through a caspase-independent mechanism. Such results are concordant with those obtained in cells treated with the combination of FP and the PKC activator PMA (29), raising the possibility that FP may exert a generalized capacity to block $p21^{WAF1/CIP1}$ induction by different classes of differentiation-inducing agents.

In contrast to $p21^{WAF1/CIP1}$, the cdki $p27^{KIP1}$ was expressed basally, but levels were minimally affected by treatment of cells with SB or PMA alone (Fig. 5*B*). However, in cells exposed to the combination of SB and FP, a rapidly migrating M_r 23,000 fragment became apparent at 12 h and was quite pronounced by 24 h, events accompanied by reduced levels of the full-length $p27^{KIP1}$ protein. Also in contrast to $p21^{WAF1/CIP1}$, cleavage of $p27^{KIP1}$ in FP/SB-treated cells was prevented by Boc-D-fmk (20 μM), indicating that this event represented a secondary process downstream of caspase activation.

The Extent of SB-induced Acetylation of Histones H3 and H4 Is Not Modified by Coadministration FP. There is considerable evidence implicating acetylation and deacetylation of histones in the regulation of transcription (38). In this regard, several classes of HDIs have been described (39), some of which, including SB, act as potent differentiation inducers (40). To investigate whether the response of U937 cells to FP/SB might involve modulation of histone acetylation, acetylated histones-3 and -4 were monitored at various intervals after exposure of cells to 1 mM SB, 100 nM FP, or the combination. As shown in Fig. 6, acetylation of histones-3 and -4 was observed in SB-treated cells, and this phenomenon was not appreciably modified by cotreatment with FP. Such findings argue against the possibility that the observed effects of the combination of FP and SB on leukemic cell maturation and apoptosis result from alterations in the extent of histone acetylation.

The Combination of FP and SB Induces Changes in the levels of Antiapoptotic Proteins through Both Caspase-dependent and -independent Mechanisms. The Bcl-2 family of apoptotic proteins contains members that promote survival (*e.g.,* Bcl-2, Bcl-X_L, and so forth), whereas others exert pro-apoptotic actions (Bak, Bax, and so forth; Ref. 41). Therefore, attempts were made to determine what effect combined treatment with SB and FP might have on levels of these proteins. In cells exposed to either SB or FP, a rapidly migrating Bcl-2 species could be faintly discerned at 12 and 24 h (Fig. 7*A*), presumably corresponding to a proapoptotic cleavage product, as described previously (42). However, cleavage of Bcl-2 was considerably more pronounced in cells exposed to both agents. Coadministration of Boc-D-fmk blocked this event, consistent with the notion that this species represents a Bcl-2 degradation product.

Recently, FP has been reported to induce a concentration-dependent reduction in expression of the antiapoptotic Bcl-2 family member Mcl-1 in B-chronic lymphocytic leukemia cells (43). When U937 cells were exposed to 100 nM FP, reductions in levels of Mcl-1 were noted at 12 and 24 h, whereas SB exerted minimal effects (Fig. 7*B*). However, the combination of FP and SB dramatically reduced Mcl-1 expression. Moreover, as observed previously in CLL cells exposed to FP alone (43), and in contrast to results involving Bcl-2, this event was not antagonized by coadministration of Boc-D-fmk. Such findings indicate that SB and FP cooperate to diminish Mcl-1 expression through a caspase-independent mechanism.

FP alone as well as the combination of FP and SB resulted in modest decreases in levels of the antiapoptotic protein XIAP (Fig. 7*C*). On the other hand, neither SB, FP, nor the combination, altered expression of the proapoptotic proteins Bax and Bak. Taken together, these findings raise the pos-

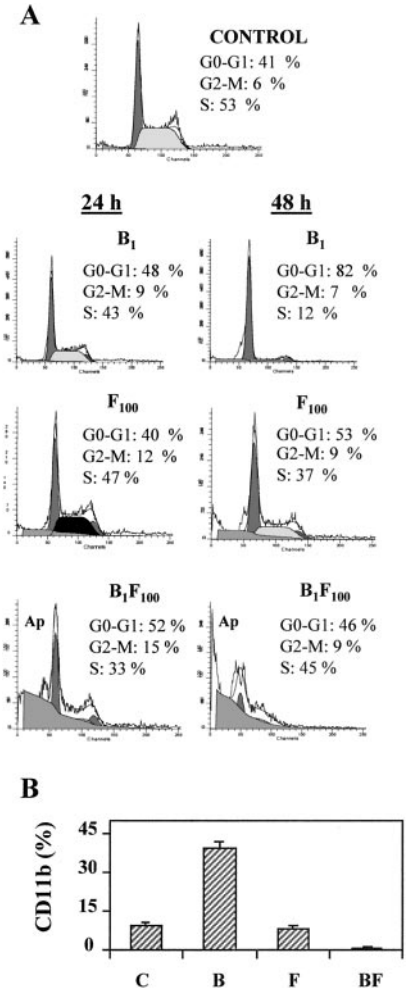

Fig. 4. *A*, cell cycle analysis of cells after exposure to FP (100 nM) and SB (1 mM) alone or in combination. U937 cells were incubated in 70% ethanol, treated with PI, and subjected to cell cycle analysis using a Becton Dickinson FACScan flow cytometer and ModFit software, as described in "Materials and Methods." The percentage values exclude the apoptotic, sub-G_0 cell population, designated *Ap*. The results of a representative study are shown; two additional experiments yielded similar results. *B*, U937 cells were incubated with 100 nM FP, 1 mM SB, or the combination for 48 h and the percentage of cells expressing the CD11b surface differentiation marker determined by flow cytometry as described in "Materials and Methods." Values represent the means for three separate experiments; *bars,* ± SD.

sibility that the combination of FP and SB may induce apoptosis, at least in part, by reducing levels of certain antiapoptotic proteins. Furthermore, they suggest that such actions may proceed through both caspase-dependent and caspase-independent mechanisms.

FP/SB Coadministration Results in pRB Dephosphorylation and Cleavage. As noted previously, both FP and SB can modulate cell cycle progression, a process that is also regulated the pRb/E2F axis (31). Therefore, attempts were made to assess the effects of this drug combination on pRb phosphorylation status. Whereas FP (100 nM) by itself did not affect levels of dephosphorylated pRb at 12 or 24 h (Fig. 8*A*), SB (1 mM), in marked contrast, induced a substantial increase in levels of the underphosphorylated form at 24 h. Coexposure to FP/SB resulted in a marked decrease in levels of full-length underphosphorylated pRb, accompanied by a clearly discernible M_r 65,000 species at both 12 and 24 h, presumably corresponding to a pRb cleavage product. This was confirmed by the observed reduction in levels of total pRb, as well as by the ability of the caspase inhibitor Boc-D-fmk to block formation of the fragment (Fig. 8*A*).

FP is known to inhibit the activities of cdks involved in pRb phosphorylation, notably cdk2 and cdk4 (44). Western blot analysis was performed to evaluate the effects of this drug combination on phosphorylation of pRb at sites specific for these cdks (Fig. 8*A*). Whereas FP or SB alone exerted only modest effects on pRb phosphorylation, the combination of FP and SB induced a dramatic reduction in expression of cdk2/4-phosphorylated pRb. Furthermore, these effects were only partially (in the case of pRb-cdk2) or minimally (in the case of pRb-cdk4) prevented by the caspase inhibitor Boc-D-fmk. In contrast, total levels of cdk2 and cdk4 proteins changed only marginally after treatment (Fig. 8*B*).

Exposure of U937 Cells to FP and SB Results in Diminished E2F/pRb Complex Formation and Down-Regulation of pRb, p130, and p107. In its hypophosphorylated form, pRb binds to members of the E2F transcription factor family, thereby inhibiting E2F-1-mediated G_1S progression (31). Therefore, the effects of FP/SB cotreatment were examined in relation to E2F-1/pRb interactions (Fig. 9*A*). After exposure to the agents alone or in combination for 8 h, pRb immunoprecipitates were obtained and treated with deoxycholate to dissociate pRb/E2F complexes. E2F liberated from pRb immunoprecipitates was then monitored by EMSA. Cells exposed to the combination of FP and SB displayed a marked reduction in E2F binding to labeled probe, reflecting a diminished pRb/E2F association (Fig. 9*A*). In contrast, Western blot analysis demonstrated the absence of changes in total E2F1 expression after all of the treatments (Fig. 9*B*). Analysis of the pRb family members (pRB, p130, and p107) demonstrated that levels of p130 and p107, as observed in the case of pRb, were substantially reduced in extracts obtained from cells exposed to both FB and SB (Fig. 9*B*). Together, these findings indicate that coexposure of leukemic cells to FB and SB results in multiple perturbations in the pRb/E2F-1 axis, including reductions in levels of pRb family members and a diminution in the association of E2F-1 with pRb.

FP/SB Treatment Induces Down-Regulation of Cyclins D1, -E, and -A. Cells traversing the cell cycle are regulated by cyclin/cdk complexes. Progression through G_1 and into S phase requires the sequential expression of cyclins D, E, and A (45). Both FP and SB have been reported to exert critical regulatory effects on cyclin expression (6, 10, 44, 46). As shown in Fig. 10, SB or FP alone induced moderate declines in cyclin D1 levels that were most noticeable 24 h after drug exposure. The ability of FP to down-regulate cyclin D1 expression has been described previously (23). However, the combination of FP and SB resulted in the total disappearance of cyclin D1 protein after only 12 h of cotreatment (Fig. 10*A*). The reduction in cyclin D1 expression was blocked by Boc-fmk, indicating that this process is caspase-dependent.

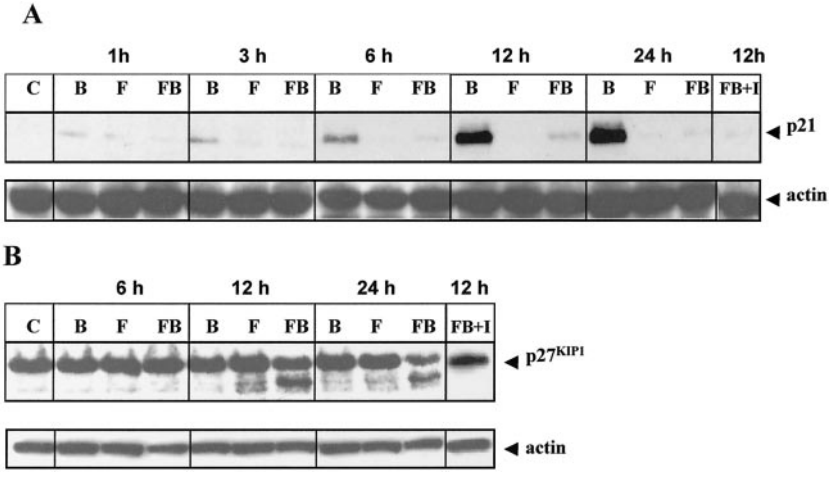

Fig. 5. *A*, Western blot analysis of the time course of the induction of $p21^{WAF1/CIP1}$ and *B*, $p27^{KIP1}$ expression in U937 cells exposed to 1 mM SB (*B*), 100 nM FP (*F*), or the combination (*FB*). After treatment for the designated interval, U937 cells were pelleted, lysed, and 30 μg of protein separated by SDS-PAGE and blotted with the appropriate antibodies. Blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. Blots designated *FP+I* correspond to lysates obtained from FP/SB-treated cells cultured in presence of the pan-caspase inhibitor BOC-D-fmk (25 μM).

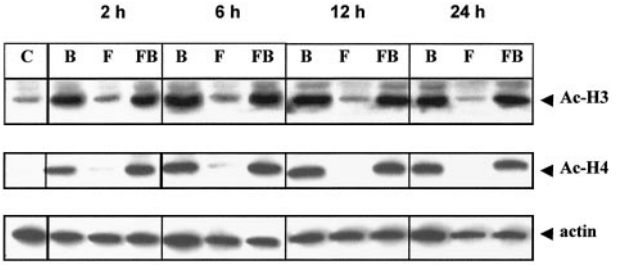

Fig. 6. Effects of FB on SB-induced acetylation of histones H3 and H4 was analyzed by Western blot. After treatment for 24 h with 100 nM FP (*F*) or 1 mM SB (*B*), cells were pelleted, and lysates were monitored for levels of acetylated histones H3 and H4 as described in "Materials and Methods." For each condition, 10 μg of extract were loaded per lane, separated on precast 4–20% Bio-Rad Gradient Gels, and blotted with the designated antibodies. Stripped blots were reblotted with an antibody to actin to control for equivalent loading and transfer. Two additional experiments yielded equivalent results.

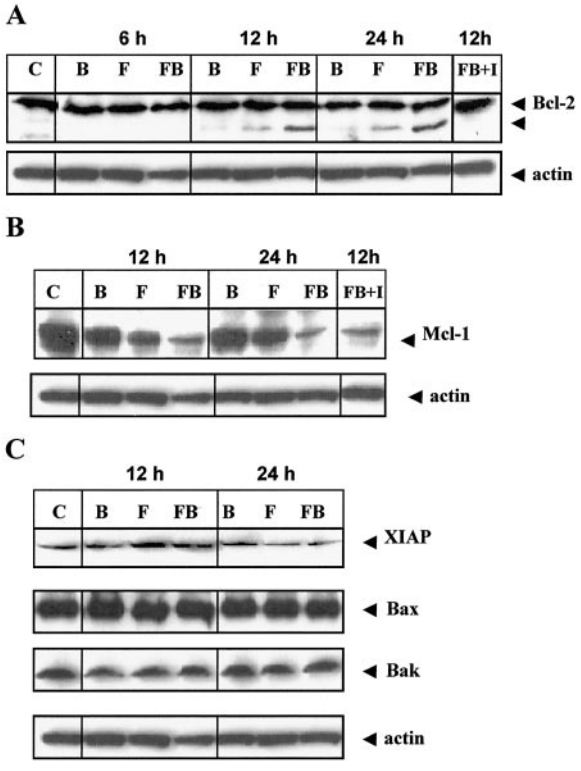

Fig. 7. *A*, Western blot analysis of the antiapoptotic proteins Bcl-2; *B*, Mcl-1; *C*, XIAP and proapoptotic proteins Bax and Bak. After treatment for the designated intervals with 100 nM FP (*F*), 1 mM SB (*B*), or the combination, U937 cells were pelleted and lysed, and 30 μg of protein were separated by SDS-PAGE and blotted with the corresponding antibodies. Membranes were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. Representative results are shown; two additional experiments showed similar results. FB + I corresponds to results obtained in FP/SB-treated cells cultured in the presence of the pan-caspase inhibitor BOC-D-fmk (25 μM).

In addition, coexposure to FP/SB substantially diminished levels of cyclin E and, to a slightly lesser extent, cyclin A (Fig. 9*B*). However, reduced expression of cyclins E and A in SB/FP-treated cells was only partially blocked by Boc-fmk treatment. Thus, cotreatment of U937 cells with FP/SB resulted in reduced expression of various cyclins involved in cell cycle progression through $G1_1S$ through both caspase-dependent and -independent mechanisms.

Blasts from AML Patients Are Sensitive to FP/SB-mediated Apoptosis. To determine whether primary AML cells might mimic the responses of continuously cultured cell lines such as U937 and HL-60 to these agents, leukemic blasts obtained from the peripheral blood of two patients with refractory AML were isolated and exposed for 24 h to various concentrations of FP and SB alone or in combination (Fig. 11*A*). Both 75 nM or 100 nM FP and 1 mM SB alone induced a modest degree of apoptosis (*e.g.,* approximately 15–20%) in the two samples. However, when cells were exposed to both 100 nM FP and 1 mM SB, the extent of apoptosis in both specimens increased dramatically (*i.e.,* to ~80%). Western blot analysis of blasts obtained from patient #1 displayed changes similar to those observed in the case of U937 cells (Fig. 11*B*). Specifically, coadministration of FP and SB resulted in a marked increase in cleavage of procaspase-3, Bcl-2, and pRb but no changes in levels of Bax. Similar results were obtained in blasts obtained from patient

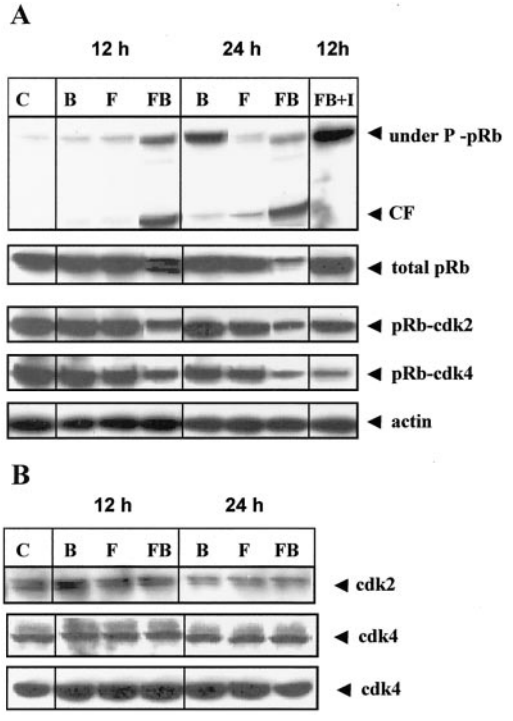

Fig. 8. In *A*, after 12- and 24-h treatment with 1 mM SB (*B*), 100 nM FP (*F*), or the combination (*FB*), U937 cells were collected and lysed, and 30 μg of protein were separated by SDS-PAGE and blotted with specific antibodies directed against the underphosphorylated form of pRb, total pRb, and pRb cdk2/4 phosphorylated sites, as well as total cdk2 and cdk4 protein. In *B,* blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. Representative results are shown; additional experiments showed similar results. FB + I corresponds to results obtained in FP/SB-treated cells cultured in the presence of the pan-caspase inhibitor BOC-D-fmk as above. *CF,* M_r 65,000 pRb cleavage product.

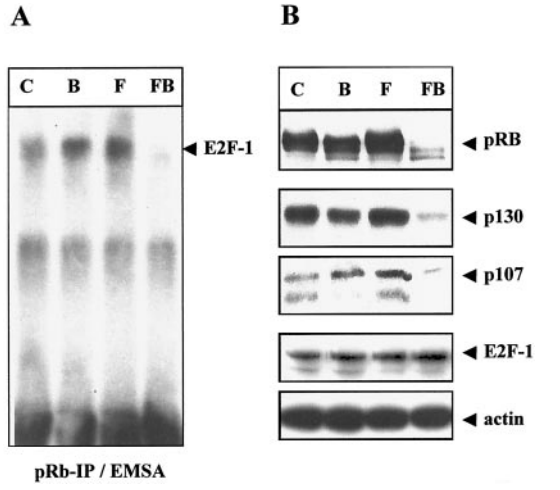

Fig. 9. In *A,* whole cells extracts were isolated from U937 cells treated for 8 h with 1 mM SB (*B*), 100 nM FP (*F*), or the combination (*FB*), and pRb immunoprecipitates were subjected to EMSA analysis. After dissociation of pRb/E2F complexes with deoxycholate, the amount of liberated E2F was determined as described in detail in "Materials and Methods." In *B,* 30 μg of protein from U937 cells treated with 1 mM SB, 100 nM FP, or the combination (*FB*) were separated by SDS-PAGE and blotted with the indicated antibodies. Representative results are shown; two additional experiments showed similar results.

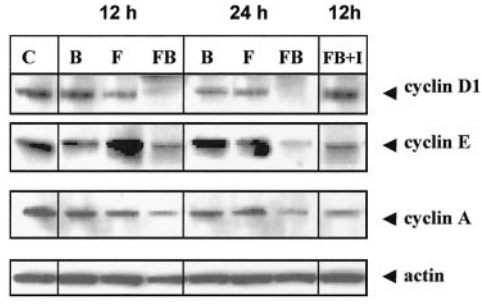

Fig. 10. U937 cells were exposed for 24 h to 1 mM SB (*B*), 100 nM FP (*F*), or the combination (*FB*). They were then pelleted and lysed, and 30 μg of protein were separated by SDS-PAGE and blotted with antibodies directed against cyclins D1, E, and A. Blots were stripped and reprobed with antibodies against actin to ensure equivalent loading and transfer. Representative results are shown; two additional experiments showed similar results. FB + I corresponds to results obtained in FP/SB-treated cells cultured in the presence of the pan-caspase inhibitor BOC-D-fmk as above.

#2 (data not shown). Thus, these findings indicate that combined treatment with SB and FP results in a marked increase in caspase activation and apoptosis in at least some primary AML blast specimens, analogous to findings in continuously cultured leukemic cell lines.

Discussion

The present results demonstrate that coadministration of the HDI and differentiation-inducing agent SB with the pharmacological cdki FP results in a dramatic increase in mitochondrial damage, caspase activation, and apoptosis in human leukemia cells. They also raise the possibility that disruption of certain cell cycle regulatory events may contribute to this phenomenon. In this context, previous studies from our laboratory and others have suggested a critical role for the cdki $p21^{WAF1/CIP1}$ in SB-related differentiation (4, 47). Interference with induction of $p21^{WAF1/CIP1}$ expression (*e.g.,* in cells stably transfected with an antisense construct) has been shown to disrupt growth arrest and subsequent maturation in cells exposed to differentiation-inducers such as PMA (8), vitamin D3 (15), and SB (4), and instead induces them to engage a default apoptotic program. Such findings are consistent with the notion that leukemic cell maturation and apoptosis represent alternative, and under some circumstances, mutually exclusive cell fates (48). Very recently, we reported that the pharmacological cdki FP opposed induction of $p21^{WAF1/CIP1}$ in human leukemia cells exposed to the PKC activator PMA, resulting a marked increase in cell death (29). However, until now, it was unclear whether this phenomenon was restricted to PKC-activating phorbol esters or could instead be extended to include an entirely different class of differentiation-inducing compounds (*e.g.,* HDIs) of which the maturation-inducing properties have also been linked to $p21^{WAF1/CIP1}$. The present results indicate that coadministration of minimally toxic concentrations of FP results in a striking increase in SB-related apoptosis in a variety of human leukemia cell

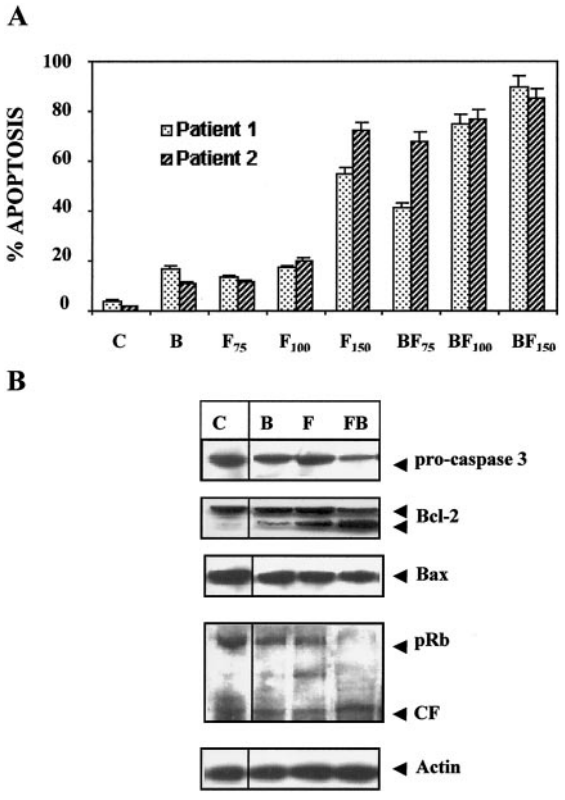

Fig. 11. In *A,* blasts were isolated from the peripheral blood of 2 patients with AML, after which they were exposed *ex vivo* to 1 mM SB; 75, 100, or 150 nM FP; or different combinations of both (*FB*) for 24 h. Apoptotic cells were identified by morphological analysis of Wright and Giemsa-stained cytospin preparations as described in "Materials and Methods." Values represent the means ±SD for triplicate determinations. *B,* Western blot analysis of caspase-3, Bcl-2, Bax, and pRb. Blasts were isolated as described and incubated during 24 h with the indicated doses of drugs, after which they were pelleted and lysed, and 30 μg of protein were separated by SDS-PAGE and blotted with antibodies directed against procaspase-3, Bcl-2, Bax, and total pRb. *CF,* M_r 65,000 pRb cleavage product. Blots were stripped and reprobed with an antibody to actin to ensure equivalent loading and transfer. A second study yielded equivalent results.

lines and that this phenomenon is accompanied by dysregulation of multiple signaling and cell cycle-related events, including inhibition of induction of $p21^{WAF1/CIP1}$. Together, these observations raise the possibility that FP may act in a generalized way to disrupt the maturation program of leukemic cells exposed to multiple differentiation-inducers, and in so doing, markedly lower the apoptotic threshold.

The susceptibility of cells to apoptosis is regulated by a large family of Bcl-2-related proteins (49), and it is tempting to speculate that the striking degree of apoptosis in SB/FP-treated cells might reflect perturbations in levels of pro- and antiapoptotic family members. For example, Bcl-2 expression has been shown to be down-regulated during SB-mediated apoptosis in MCF-7 breast cancer cells (50) and in RG/C2 and BH/C1 human colonic adenoma cell lines (51). However, whereas in the present study a significant change in total Bcl-2 expression was not observed in FP/SB-treated cells, a clear increase in expression of a Bcl-2 cleavage product was noted. Moreover, this phenomenon was blocked by caspase inhibition. In this context, Bcl-2 is a substrate of caspase-3 that undergoes cleavage to a proapoptotic M_r 23,000 fragment, which may serve to amplify the caspase cascade (52). Furthermore, the M_r 23,000 fragment is known to promote cytochrome *c* release (53), a phenomenon that may have contributed to the time-dependent increase observed in cells coexposed to FP/SB.

Other events that might have played a role in amplification of apoptosis in cells exposed to SB/FP include Bid cleavage and down-regulation of the antiapoptotic proteins Mcl-1 and XIAP. It is noteworthy that in FP/SB-treated cells, caspase-8 activation was observed at the 12-h interval, in parallel with the activation/cleavage of Bid. It is known that activation of caspase-8 results in Bid activation, which then translocates to mitochondria and induces cytochrome *c* release (54). Thus, Bid cleavage could also contribute to the time-dependent increase of cytochrome *c* release observed in FP/SB-treated cells. Other proteins potentially involved in regulation of FP/SB-induced apoptosis include Mcl-1 and XIAP. FP has been reported previously to induce a marked caspase-independent reduction in Mcl-1 and XIAP protein levels in primary CLL cells (43), analogous to the present findings. In view of these observations, the possibility that enhanced down-regulation of Mcl-1 and XIAP in cells exposed to both SB and FP contributes to the marked increase in apoptosis appears plausible. Whereas the mechanism by which this phenomenon occurs is unclear, the possibility that down-regulation of these proteins, as well as $p21^{WAF1/CIP1}$, involves transcriptional repression after FP-associated DNA duplex formation (55) must be considered. In this context, recent evidence that FP-related transcriptional repression involves inhibition of cdk9/cyclin T may be pertinent (56, 57).

It is tempting to attribute enhanced apoptosis in SB/FP-treated cells to dysregulation of cell cycle-related events. For example, it has been reported that induction of the endogenous cdki $p21^{WAF1/CIP1}$ (or lack thereof) determines whether a cell undergoes maturation or apoptosis in response to a differentiation stimulus (*e.g.,* PMA; Ref. 58). In this regard, several studies have demonstrated that $p21^{WAF1/CIP1}$ may exert an antiapoptotic effect distinct from its cell cycle inhibitory actions, *i.e.,* by forming a complex with and inhibiting the activity of procaspase-3 (10). Thus, FP, by directly inhibiting cdks, may render increased expression of cdkis such as $p21^{WAF1/CIP1}$ by SB redundant. Alternatively, FP may directly block $p21^{WAF1/CIP1}$ induction, possibly through DNA interactions as noted above (55). In any case, the loss of a putative $p21^{WAF1/CIP1}$ cytoprotective effect could potentially convert SB from a differentiation stimulus to an apoptotic one. Another possibility is that enhanced apoptosis in SB/FP-treated cells may stem from down-regulation of the cdki $p27^{KIP1}$, which has been shown previously to mediate growth arrest in G_1 and to confer resistance to apoptosis induced by anticancer agents (59). Furthermore, cleavage of $p27^{KIP1}$ has been implicated in the induction of apoptosis in certain cell types (*e.g.,* endothelial cells; Ref. 60). However, in contrast to the case of $p21^{WAF1/CIP1}$, $p27^{KIP1}$ down-regulation was blocked by the caspase inhibitor Boc-D-fmk, arguing against

a primary role for down-regulation of this cdki in the initiation of SB/FP-mediated apoptosis. Nevertheless, the possibility that cleavage/down-regulation of $p27^{KIP1}$ serves to amplify the apoptotic process cannot be excluded.

Several FP-mediated disruptions in cell cycle arrest/and the pRb/E2F axis may also have contributed to potentiation of leukemic cell apoptosis after SB exposure. For example, FP/SB-treated U937 cells failed to undergo growth arrest but instead engaged an apoptotic cell death program. The pRb/E2F axis is a critical regulator of the G_1-S transition (31), and arrest of cells in G_1 is required for normal maturation (28). Dephosphorylated pRb binds to and inactivates the cell cycle transcription factor E2F, thereby repressing gene transcription required for this transition (61). The effects of FP on this process are likely to be complex in that direct actions (*e.g.,* cdk inhibition) would favor pRb dephosphorylation, whereas inhibition of $p21^{WAF1/CIP1}$ induction would oppose this process. However, coexposure of SB-treated cells to FP ultimately resulted in a net reduction in expression of underphosphorylated pRb, which in all likelihood reflected a combination of caspase-mediated cleavage of pRb as well as down-regulation of $p21^{WAF1/CIP1}$. Consistent with this notion, a reduction was noted in E2F1 detected by EMSA in pRb immunoprecipitates. Similarly, levels of other members of the pRb family (*e.g.,* p130 and p107) were also markedly reduced. Loss of pRb has been shown to lead to both p53-dependent and -independent apoptosis (62), and inappropriate activation of E2F is a potent inducer of apoptosis (63). Furthermore, it has been shown recently that E2F induces the expression of several key regulators of apoptosis, including apaf-1 and effector caspases such as caspase-3 and -7 (64). Thus, deregulated E2F-1 activity accompanying reduction in levels of underphosphorylated pRb could contribute to the extensive apoptotic response observed in cells treated with the combination FP and SB.

Coexposure of SB-treated cells to FP also induced changes in levels of expression of several cyclins. Cyclins are critical regulators of the transition between various phases of the cell cycle (45). The D-type cyclins are the first cyclins to be expressed as cells in G_0 are stimulated to enter the cell cycle (11). Through activation of E2F, cyclin E is subsequently induced during the progression of cells into G_1 (65). Cyclin A, which is also regulated in part by E2F (66), is required for the onset of DNA replication during S phase (67). Both FP and SB have been shown to modulate the expression of several cyclins. For example, Carlson *et al.* (23) demonstrated that FP induced an early reduction on cyclin D protein levels in MCF-7 human breast carcinoma cells. In addition, we have reported that SB also reduces cyclin D1 expression in U937 cells (4), and similar observations have been reported in other cell models (6, 46). In view of these findings, it is not surprising that the combination of SB and FP dramatically diminished cyclin D1 levels as early as 12 h after drug exposure. However, the ability of Boc-D-fmk to oppose this process suggests that this phenomenon is caspase-dependent, at least in part. In addition, SB has also been found to modulate the expression of cyclins E and A in 3T3 fibroblasts (10) and in U937 cells (4). In the present studies, the combination of SB and FP resulted in a very pronounced reduction in cyclin E and A expression in U937 cells through a process that was at least partially caspase-independent. Taken together, these findings raise the possibility that altered expression of key cell cycle regulatory molecules, including cyclins D1, E, and A, may additionally promote activation of the apoptotic cascade and/or contribute to its amplification.

In addition to dysregulation of cell cycle and apoptotic proteins involved in the control of apoptosis, the possibility also arises that FP and SB interact directly at the mitochondrial level. For example, the ability of FP to induce mitochondrial damage (*e.g.,* cytochrome *c* release) has been described in U937 cells (33), as has the capacity of SB to trigger mitochondrial injury in Y79 retinoblastoma cells (68). Very recent reports suggest that BH3 domain-only proteins such as Bid interact with the proapoptotic, multidomain proteins Bax and Bak to promote cytochrome *c* release and that these interactions are opposed by Bcl-2 and Bcl-xL (69) Additional studies will be required to establish whether, and to what extent, promotion of mitochondrial dysfunction through such mechanisms contributes to the pronounced apoptosis that occurs in leukemic cells coexposed to these agents.

Finally, it is important to note that evidence of enhanced apoptosis, including caspase-3 activation, Bcl-2 cleavage, and pRb degradation, was also observed in two primary AML blast samples after *ex vivo* exposure to the combination of FP and SB. The appearance of apoptotic cells in the peripheral blood of leukemic patients undergoing chemotherapy has been well documented (70), and it is conceivable that these agents might exert similar effects when administered *in vivo*. However, whether the combination of FP and SB promotes apoptosis in a larger series of primary AML specimens and whether the observed *in vitro* interactions occur in the *in vivo* setting remain to be determined.

In summary, the present findings indicate that the combination of the cdki FP with the HDI and differentiation-inducer SB does not promote cellular maturation but instead results in a dramatic induction of apoptosis in U937 cells as well as other human leukemia cell lines. This phenomenon is associated with multiple perturbations, including early alterations in mitochondrial function and a striking inhibition by FP of SB-mediated induction of the cdki $p21^{WAF1/CIP1}$. These phenomena are also accompanied by a variety of subsequent events, including proteolytic cleavage of $p27^{Kip1}$, Bcl-2, and Bid, disruption of the pRb/E2F axis, and diminished expression of cell cycle-related proteins, including cyclins D_1, E and A, that may collectively serve to amplify the apoptotic response. A hypothetical model summarizing these events is shown in Fig. 12. Such findings, as well as the observation that the combination of SB and FP potently induced apo ptosis in two patient-derived AML samples, along with the recent introduction of FP and HDIs into the clinical arena (22, 71), raise the possibility that this approach, and perhaps others capable of disrupting the $p21^{WAF1/CIP1}$ and pRB/E2F axes, may represent a novel therapeutic strategy in leukemia. Whether such a strategy will prove sufficiently selective by sparing normal host target tissues remains to be determined. Accordingly, efforts to address this issue, and to extend

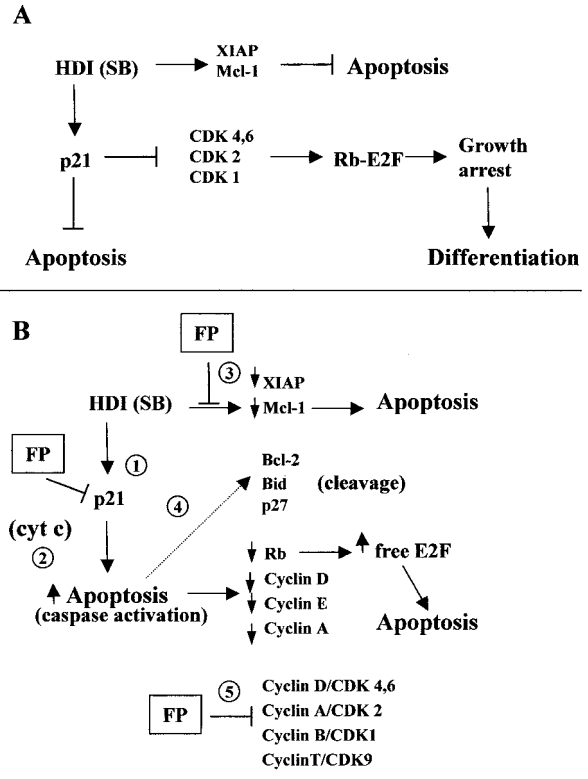

Fig. 12. Hypothetical model of SB-FP interactions in human leukemia cells. Under ordinary circumstances (*A*), exposure of leukemic cells to SB results in up-regulation, at the transcriptional level, of the cdki $p21^{WAF1/CIP1}$, which inhibits multiple cyclin/cdk complexes and may also exert direct anti-apoptotic actions. cdk inactivation leads in turn to pRb dephosphorylation, which then binds and inactivates E2F, resulting in transcriptional repression of cell cycle progression genes, culminating in cellular maturation. In addition, SB may also induce expression antiapoptotic proteins, such as Mcl-1 and XIAP, which limit the extent of apoptosis that occurs during the differentiation process. However, coadministration of FP (*B*) may perturb these events in multiple ways including (*1*) down-regulation of $p21^{WAF1/CIP1}$ and abrogation of its antiapoptotic effects; (*2*) direct promotion of cytochrome *c* release; and (*3*) down-regulation of antiapoptotic proteins, such as Mcl-1 and XIAP. The resulting caspase activation (*4*) may lead in turn to cleavage of Bcl-2 into a proapoptotic fragment, activation of Bid, cleavage of $p27^{KIP1}$, diminished expression of cyclins, and pRb degradation. The latter event results in "inappropriate" E2F activation and potentiation of cell death. Finally, FP directly inhibits the activity of multiple cyclin/cdk complexes, one of which (cyclin T/cdk9) may possibly be involved in the transcription of p21, Mcl-1, and cyclins E and A. The net effect of these multiple FP-related perturbations is to shift leukemic cells away from the "normal" SB-associated maturation program and toward an alternative cell death fate.

these observations to other clinically relevant HDIs are currently underway.

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