Extrinsic pathway- and cathepsin-dependent induction of mitochondrial dysfunction are essential for synergistic flavopiridol and vorinostat lethality in breast cancer cells

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Abstract

The present studies have determined whether interactions between the cyclin-dependent kinase inhibitor flavopiridol and the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA; vorinostat; Zolinza) occur in breast cancer cells. MDA-MB-231 and MCF7 cells were treated with flavopiridol (25–100 nmol/L) and vorinostat (125–500 nmol/L) in vitro, and mechanisms of cell killing were determined. Concurrent treatment of cells with flavopiridol and vorinostat or treatment of cells with flavopiridol followed by vorinostat promoted cell killing in a greater than additive fashion. Similar data were obtained with the CDK inhibitor roscovitine. Flavopiridol suppressed c-FLIP-L/s and BCL-xL expression, whereas vorinostat reduced expression of BCL-xL, and combined exposure to flavopiridol and vorinostat reduced MCL-1 and X-chromosome–linked inhibitor of apoptosis protein (XIAP) levels. Pharmacologic or genetic inhibition of c-FLIP-L/s, BCL-xL, and XIAP expression and protected cells against flavopiridol/vorinostat lethality. Overexpression of c-FLIP-L/s and BCL-xL abolished the lethality of flavopiridol/vorinostat. Collectively, these data argue that flavopiridol enhances the lethality of vorinostat in breast cancer cells in part through the inhibition of Akt and ERK1/2 function, leading to reduced expression of multiple inhibitors of the extrinsic and intrinsic apoptosis pathways, as well as activation of cathepsin protease-dependent pathways. [Mol Cancer Ther 2007;6(12):3101–12]

Introduction

Histone acetylation plays a significant role in the control of transcription in multiple cell types (1). Histone acetylation is reciprocally regulated by histone deacetylases (HDAC) and histone acetyltransferase enzymes, with the acetylation of histones permitting chromatin to assume a relaxed conformation, thereby promoting transcription (2, 3). Histone deacetylase inhibitors (HDACIs) are a group of agents that block histone deacetylation, thus altering the rates of transcription of many different genes, including those regulating cell survival, the cell cycle, and cellular differentiation (4). At low concentrations in vitro, HDACIs such as sodium butyrate and suberoylanilide hydroxamic acid (SAHA; vorinostat; Zolinza) have been shown to induce cellular differentiation (5, 6). However, higher in vitro concentrations of vorinostat as a single agent induce apoptosis (7). Clinical trials of butyrate derivatives have been done, and phase I and II trials using vorinostat are in progress (8, 9). Vorinostat was approved by the U.S. Federal Drug Administration for at least one indication (cutaneous T-cell lymphoma) in October 2006.

Flavopiridol (NSC 649890) is a semisynthetic alkaloid that inhibits to varying degrees all known cyclin-dependent kinases (CDK), including the cyclin T/CDK9 transcriptional regulatory complex [positive transcription elongation factor-b (PTEF-b); refs. 10, 11]. Inhibition of CDK9 results in the phosphorylation of the carboxyl-terminal domain of RNA Pol II and reduced levels of transcription (12). Flavopiridol was the first CDK inhibitor to enter clinical trials (13). In vitro, clinically relevant low concentrations...
(<200 nmol/L) of flavopiridol induce G1 arrest in tumor cells and variably trigger apoptosis (14, 15). Flavopiridol toxicity correlates with the transcription repression of various genes that promote cell survival, including those encoding short-lived proteins such as MCL-1 (16, 17). Studies from several laboratories have linked some of the lethal actions of flavopiridol in leukemic cells to inhibition of IκB kinases and inactivation of the transcription factor nuclear factor-κB (NF-κB), a transcription factor involved in diverse cellular processes, including cell survival, proliferation, and differentiation (18). Treatment of cells with flavopiridol has also been shown to inhibit the activities of many signal transduction pathways that are frequently associated with cell survival and the regulation of cell survival protein expression, e.g., AKT (17, 19, 20).

Multiple studies have shown that coadministration of flavopiridol with HDACIs increases mitochondrial dysfunction, leading to subsequent induction of apoptosis in a variety of cancer cell types, particularly leukemic cells (17, 19–21). It has been postulated that the interactions between these agents may stem from flavopiridol-mediated transcriptional repression of short-lived antiapoptotic cell survival proteins such as MCL-1 and X-chromosome–linked inhibitor of apoptosis protein (XIAP), as well as suppression of NF-κB function (17, 19, 20, 22).

Although it is known that vorinostat, administered at relatively high concentrations (e.g., 5 μmol/L) induce apoptosis in certain epithelial tumors (i.e., breast cancer cells), the ability of flavopiridol and vorinostat to modulate the survival of such cells, particularly at lower, more pharmacologically achievable concentrations, has not yet been addressed. The present studies were undertaken to determine whether flavopiridol and vorinostat interact synergistically in a mammary tumor cell model and, if so, to gain insights into the mechanism(s) of cell death. The present results indicate that concomitant treatment of cells with relatively low (i.e., sub-micromolar) concentrations of vorinostat and flavopiridol induce a greater than additive induction of cell killing in short-term viability assays and an at least additive suppression of colony formation. Notably, flavopiridol lethality was suppressed by pan-inhibition of caspase function and by the loss of cathepsin B expression. Furthermore, cell killing by this regimen involved a caspase-8– and cathepsin-related induction of BID-dependent mitochondrial dysfunction. Collectively, these findings argue that the mechanisms by which flavopiridol and vorinostat interact to kill mammary carcinoma cells share certain features with, but also exhibit distinct differences from, those previously described in leukemic cells.

**Materials and Methods**

**Materials**

Phospho–/total extracellular signal-regulated kinase 1/2 (ERK1/2), phospho–/total c-Jun-NH2 kinase 1/2 (JNK1/2), phospho–/total p38 mitogen-activated protein kinase (MAPK), anti-S473 AKT, and total AKT antibodies were purchased from Cell Signaling Technologies. All the secondary antibodies [anti-rabbit horseradish peroxidase (HRP), anti-mouse HRP, and anti-goat HRP] were purchased from Santa Cruz Biotechnology. Vorinostat and flavopiridol were kindly supplied Merck and Sanofi-Aventis, respectively, through the Cancer Treatment and Evaluation Program, National Cancer Institute. The JNK inhibitor peptide (JNK IP) was supplied by Calbiochem as powder, dissolved in sterile DMSO, and stored frozen under light-protected conditions at −80°C. Enhanced chemiluminescence (ECL) kits were purchased from Amersham ECL system and NEN Life Science Products. Trypsin-EDTA, RPMI medium, penicillin-streptomycin were purchased from Life Technologies BRL. BAX/BAK−/−, BIM−/−, and BID−/− fibroblasts were kindly provided by Dr. S. Korsmeyer (Harvard University, Boston, MA). Transformed protein kinase R (PKR)-like endoplasmic reticulum (PERK)−/− cells were a kind gift from the Ron Laboratory (Skirball Institute, New York University School of Medicine, New York, NY). Immortalized cathepsin B−/− fibroblasts and matched wild-type fibroblasts were kindly supplied by Christoph Peters and Thomas Reinheckel (Medizinische Universitaetsklinik Freiburg, Freiburg, Germany) and Paul Saftig (Christian-Albrechts-Universitaet Kiel, Kiel, Germany). The activated MAP/ERK kinase 1 (MEK1) EE adenovirus was kindly provided by Dr. J. Moltken (University of Cincinnati, Cincinnati, OH), respectively. Other reagents were of the highest quality commercially available as described previously (23).

**Methods**

**Culture and In vitro Exposure of Cells to Drugs.** MDA-MB-231 and MCF7 cells were cultured at 37°C (5% v/v CO2) in vitro using RPMI supplemented with 10% (v/v) FCS. In vitro flavopiridol/vorinostat treatment were from 100 mmol/L stock solutions of each drug, and the maximal concentration of vehicle (DMSO) in media was 0.02% (v/v). For colony formation assays, cells were plated at low density (250–2,000 cells per dish), and 12 h after plating, cells were treated with the drugs in the order stated and at the concentrations stated in the figure and figure legend. Ten to fourteen days after exposure, plates were washed in PBS, fixed with methanol, and stained with a filtered solution of crystal violet (5% w/v). After washing with tap water, the colonies were counted both manually (by eye) and digitally using a ColCount TM plate reader. Data presented are the arithmetic mean (± SE) from both counting methods from multiple studies.

**3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assays to Assess Cell Growth and Viable Cell Number after Drug Exposure.** Cells growing in log phase were plated in 12-well plates (10,000 cells per well) and, 24 h after plating, treated with the indicated drugs in the sequence and at the concentrations described in the figure and figure legend. At the indicated time points, the medium was removed, and a fresh medium containing 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent was added to each well. The cells were incubated at 37°C for 4 h, and then an equal volume of DMSO was added to each well and mixed.
thoroughly. The absorbance from the plates was read on a microplate reader at 595 nm.

**Cell Treatments, SDS-PAGE, and Western Blot Analysis.** Cells were treated with flavopiridol or vorinostat in either a sequence-dependent fashion or concomitantly, as indicated in the figure legend. For SDS-PAGE and immunoblotting, cells were lysed in either a non-denaturing lysis buffer and prepared for immunoprecipitation or in whole-cell lysis buffer [0.5 mol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromophenol blue], and the samples were boiled for 30 min. After immunoprecipitation, samples were boiled in whole-cell lysis buffer. The boiled samples were loaded onto 10% to 14% SDS-PAGE, and electrophoresis was run overnight. Proteins were electrophoretically transferred onto 0.22 μm nitrocellulose and immunoblotted with various primary antibodies against different proteins. All immunoblots were visualized by ECL. For presentation, immunoblots were digitally scanned at 600 dpi using Adobe Photoshop 7.0 and their color was removed, and figures were generated in MicroSoft PowerPoint. Densitometric analysis for ECL immunoblots were done using a Fluorochem 8800 Image System and the respective software (Alpha Innotech Corporation, San Leandro, CA), and band densities were normalized to that of a total protein loading control.

**Recombinant Adenoviral Vectors; Infection *In vitro*.** We generated and purchased previously described recombinant adenoviruses to modulate p21 expression and to express constitutively activated and dominant-negative AKT and MEK1 proteins, dominant-negative caspase-9, XIAP, c-FLIP-S, cytokine response modifier gene A (CRM A), and BCL-XL (Vector Biolabs). Cells were infected with these adenoviruses at an approximate multiplicity of infection (MOI) of 50. Cells were further incubated for 24 h to ensure adequate expression of transduced gene products before drug exposures.

**Detection of Cell Death by Trypan Blue and Flow Cytometry Assays.** Cells were harvested by trypsinization with trypsin/EDTA for ~10 min at 37°C. As some apoptotic cells detached from the culture substratum into the medium, these cells were also collected by centrifugation of the medium at 1,500 rpm for 5 min. The pooled cell pellets were resuspended, and a fraction of the suspension was centrifuged in a cytopipette (Cytopsin 3, Shandon Inc.). For Wright Giemsa staining, the slides were fixed and stained in Diff-Quik™ Stain Set (Dade Diagnostics of P.P. Inc.) according to the manufacturer’s instructions and viewed under a light microscope. Nuclear and total cellular morphology was evaluated. Giemsa staining was used to identify total cell numbers and total numbers of apoptotic and non-apoptotic manifestations of cell killing. Five hundred cells from several randomly chosen fields were counted, and the number of apoptotic cells was counted and expressed as a percentage of the total number of cells counted.

**Plasmid Transfection.** Plasmid DNA (0.5 μg/total plasmid transfected) was diluted into 50 μL of RPMI growth media that lacked supplementation with fetal bovine serum (FBS) or with penicillin-streptomycin. LipofectAMINE 2000 reagent (1 μL, Invitrogen) was diluted into 50 μL growth media that lacked supplementation with FBS or with penicillin-streptomycin. The two solutions were then mixed together and incubated at room temperature for 30 min. The total mixture was added to each well (four-well glass slide or 12-well plate) containing 200 μL growth media that lacked supplementation with FBS or with penicillin-streptomycin. The cells were incubated for 4 h at 37°C, after which time the media was replaced with RPMI growth media containing 5% (v/v) FBS and 1x penicillin-streptomycin.

**Microscopy for Acidic Endosomes and LC3-GFP.** Transfected cells were pretreated with 3-methyladenine (5 mmol/L, Sigma) 30 min before flavopiridol/vorinostat exposure and then cultured for 12 to 48 h. Cells were then stained with Lysotracker Red Dye (Invitrogen) at the indicated time points for 20 min. Lysotracker Red Dye–stained cells were visualized immediately after staining on a Zeiss Axiovert 200 microscope using the rhodamine filter. LC3-GFP–transfected cells were visualized at the indicated time points on the Zeiss Axiovert 200 microscope using the FITC filter.

**Data Analysis.** Comparison of the effects of various treatments was done using one-way ANOVA and a two tailed Student’s t test. Differences with a P value of <0.05 were considered statistically significant. Experiments shown are the means of multiple individual points from multiple experiments (± SE). Characterization of synergistic and antagonistic interactions in cells exposed to a range of vorinostat and flavopiridol concentrations administered at a fixed ratio was done using median dose-effect analysis in conjunction with a commercially available software program (CalcuSyn, Biosoft).

**Results**

**Flavopiridol and Vorinostat Interact in a Synergistic Manner to Promote Mammary Tumor Cell Death *In vitro*.** The impact of combined exposure of breast cancer cells to the CDK inhibitor flavopiridol and the HDACI vorinostat was first investigated. In multiple short-term cell viability assays, including annexin–propidium iodide, terminal nucleotidyl transferase–mediated nick end labeling...
(TUNEL), Geimsa, and trypan blue exclusion, simultaneous combined exposure of MDA-MB-231, MCF7, and MDA-MB-468 cells to flavopiridol and vorinostat resulted in a greater than additive induction of short-term cell killing compared with either drug individually, which was synergistic as determined by median dose-effect analyses, with combination index (CI) values consistently <1.00 (Fig. 1A–E, data not shown).
Flavopiridol and Vorinostat Interact in a Sequence-Dependent Manner to Cause Mammary Carcinoma Cell Death

The impact of altering the sequence of drug exposure on cell proliferation using MTT assays and on cell survival using colony formation assays was then investigated. In general agreement with the short-term cell viability data, treatment with flavopiridol caused more growth suppression than vorinostat in MTT assays and more inhibition of colony formation than vorinostat in colony formation assays (Fig. 2A–D). Treatment of MDA-MB-231 and MCF7 cells with flavopiridol and vorinostat for 96 h, either concomitantly or with the sequence flavopiridol followed by vorinostat exposure, suppressed cell growth in MTT assays and colony formation in clonogenic assays, to an extent that was significantly greater than the actions of the individual agents administered alone (Fig. 2A–D). However, surprisingly, treatment of cells with vorinostat (500 nmol/L), before flavopiridol (100 nmol/L), slightly reduced the growth suppressive and lethal effects of flavopiridol. Parallel studies with another CDK inhibitor, roscovitine, generated data that were very similar to that generated using flavopiridol (refs. 24, 25; data not shown).

Activation of the Extrinsic Pathway Plays a Central Role in the Toxicity of Flavopiridol and Vorinostat as Individual Agents, and in Combination, in Breast Cancer Cells

Subsequent studies then examined additional mechanisms by which flavopiridol and vorinostat interacted to kill mammary carcinoma cells using both pharmacologic and genetic approaches. In MDA-MB-231 cells, treatment with a caspase-9 inhibitor (LEHD) or expression of dominant-negative caspase-9 weakly altered the toxicity of either flavopiridol or vorinostat administered individually, but significantly attenuated the toxicity of combined flavopiridol and flavopiridol treatment (Fig. 3A and B). Furthermore, expression of the viral caspase-8 inhibitor, CRM A, or treatment with a caspase-8 inhibitor (IETD) modestly suppressed flavopiridol and vorinostat toxicity, but again, significantly suppressed the lethality of combined flavopiridol and flavopiridol treatment, and to the same extent as incubation with a pan-caspase inhibitor.

Treatment with flavopiridol and vorinostat did not promote the appearance of autophagic vacuoles in MDA-MB-231 cells (Fig. 3B, inset). In MCF7 cells, inhibition of caspase function did not modify the toxicity of flavopiridol, but suppressed vorinostat lethality (Fig. 3C). Inhibition of caspase-9 function did not modify vorinostat toxicity, but partially suppressed lethality in cells treated with vorinostat and flavopiridol. Finally, inhibition of caspase-8 suppressed vorinostat toxicity and abolished the interaction between vorinostat and flavopiridol to the same extent as a pan-caspase inhibitor (Fig. 3C). Collectively, these data argue that first, a significant component of flavopiridol lethality in these breast cancer cell lines is caspase independent, and second, that a significant mode of cell killing induced by combined exposure to vorinostat and flavopiridol is caspase-8 dependent.

Deletion of Cathepsin B, BID, and BAX/BAK Function Suppresses the Lethality of Flavopiridol and Vorinostat Treatment

To assess the mechanisms underlying vorinostat/flavopiridol interactions more rigorously, transformed mouse embryonic fibroblasts (MEF), in which various proapoptotic genes had been deleted, were employed. Flavopiridol and vorinostat caused a similar induction of cell killing in wild-type MEFs as previously observed in breast cancer cells (Fig. 4A and B). Interestingly, loss of expression of PERK, which is intimately involved in mediating the ER stress response (26), enhanced the lethality of flavopiridol, but did not significantly alter the survival of cells exposed to vorinostat/flavopiridol. In contrast, loss of BAX/BAK and BID function significantly suppressed the lethality of flavopiridol and abolished the potentiation of flavopiridol lethality by vorinostat (Fig. 4A). Notably, treatment with flavopiridol and vorinostat promoted BID cleavage (Fig. 4B, inset), consistent with a requirement for the extrinsic pathway in the lethality of this regimen. On the other hand, loss of BIM function did not alter the lethality of flavopiridol and vorinostat treatment, whereas loss of function of the serine protease cathepsin B function profoundly suppressed the toxicity of both flavopiridol and flavopiridol/vorinostat (Fig. 4B). In view of these findings, breast cancer cells were incubated with a claimed...
Flavopiridol and vorinostat interact in a sequence-dependent manner to cause mammary carcinoma cell death. A and B, human mammary carcinoma cells. MDA-MB-231 (A) and MCF7 (B) were plated as in Methods and, 24 h after plating, treated with vehicle control (VEH, DMSO) or with flavopiridol (FP, 100 nmol/L) or with vorinostat (Vor, 500 nmol/L). Cells were drug treated either concurrently (con), with flavopiridol 24 h before vorinostat (FP → Vor.) or with vorinostat 24 h before flavopiridol (Vor. → FP). Cell numbers for all treatment conditions were determined in triplicate 96 h after drug exposure using MTT assays, where the data for vehicle control treatment for comparison to flavopiridol effects were defined as 1.00, and the data for vorinostat treatment for comparison to flavopiridol effects were defined as 1.00 (± SE, n = 3); *, P < 0.05, greater than additive reduction in cell numbers for the combined exposure compared with individual drug treatments; †, P < 0.05, less suppression of cell numbers than in cells treated with both drugs concurrently. C and D, human mammary carcinoma cells. MDA-MB-231 (C) and MCF7 (D) were plated as single cells as in Methods and, 14 h after plating, treated with vehicle control (DMSO) or with flavopiridol (100 nmol/L) or with vorinostat (500 nmol/L). Cells were drug treated either concurrently (con), with flavopiridol 24 h before vorinostat (FP → Vor.) or with vorinostat 24 h before flavopiridol (Vor. → FP). Ninety-six hours after the start of drug treatment, for all treatment conditions, media containing drugs was removed, cells were washed with media, and fresh media was added; cells were cultured for an additional 10 days. Colonies were fixed and stained, and colony numbers were determined in triplicate, wherein the data for vehicle control treatment for comparison to flavopiridol effects were defined as 1.00, and the data for vorinostat treatment for comparison to flavopiridol effects were defined as 1.00 (± SE, n = 3); *, P < 0.05, greater than additive reduction in colony numbers for the combined exposure compared with individual drug treatments; †, P < 0.05, less suppression of colony numbers than in cells treated with both drugs concurrently.

Figure 2.

Flavopiridol and vorinostat interact in a sequence-dependent manner to cause mammary carcinoma cell death. A and B, human mammary carcinoma cells. MDA-MB-231 (A) and MCF7 (B) were plated as in Methods and, 24 h after plating, treated with vehicle control (VEH, DMSO) or with flavopiridol (FP, 100 nmol/L) or with vorinostat (Vor, 500 nmol/L). Cells were drug treated either concurrently (con), with flavopiridol 24 h before vorinostat (FP → Vor.) or with vorinostat 24 h before flavopiridol (Vor. → FP). Cell numbers for all treatment conditions were determined in triplicate 96 h after drug exposure using MTT assays, where the data for vehicle control treatment for comparison to flavopiridol effects were defined as 1.00, and the data for vorinostat treatment for comparison to flavopiridol effects were defined as 1.00 (± SE, n = 3); *, P < 0.05, greater than additive reduction in cell numbers for the combined exposure compared with individual drug treatments; †, P < 0.05, less suppression of cell numbers than in cells treated with both drugs concurrently. C and D, human mammary carcinoma cells. MDA-MB-231 (C) and MCF7 (D) were plated as single cells as in Methods and, 14 h after plating, treated with vehicle control (DMSO) or with flavopiridol (100 nmol/L) or with vorinostat (500 nmol/L). Cells were drug treated either concurrently (con), with flavopiridol 24 h before vorinostat (FP → Vor.) or with vorinostat 24 h before flavopiridol (Vor. → FP). Ninety-six hours after the start of drug treatment, for all treatment conditions, media containing drugs was removed, cells were washed with media, and fresh media was added; cells were cultured for an additional 10 days. Colonies were fixed and stained, and colony numbers were determined in triplicate, wherein the data for vehicle control treatment for comparison to flavopiridol effects were defined as 1.00, and the data for vorinostat treatment for comparison to flavopiridol effects were defined as 1.00 (± SE, n = 3); *, P < 0.05, greater than additive reduction in colony numbers for the combined exposure compared with individual drug treatments; †, P < 0.05, less suppression of colony numbers than in cells treated with both drugs concurrently.
relatively specific inhibitor of cathepsin B followed by treatment with vorinostat and flavopiridol. These studies revealed that inhibition of cathepsin protease function in breast cancer cells was at least as potent in suppressing vorinostat/flavopiridol toxicity as inhibition of caspase protease function (Fig. 4C). Finally, inhibition of either caspase-8 and/or cathepsin function suppressed drug-induced BID cleavage in breast cancer cells (Fig. 4C, inset). Collectively, these findings argue that the activation of the extrinsic apoptotic pathway as well as lysosomal serine proteases play a significant role in the lethality of the flavopiridol/vorinostat regimen in breast cancer cells.

**Deletion of Cathepsin B, BID, and BAX/BAK Function Suppresses the Lethality of Flavopiridol and Vorinostat Treatment**

In view of the significant correlation between the activation of caspase-8 and the induction of cell death, additional studies examined the expression of caspase-8 regulatory molecules and signaling pathways in drug-treated MDA-MB-231 cells. Forty-eight hours after combined flavopiridol/vorinostat exposure, levels of the cleaved forms of caspase-3 and poly(ADP-ribose) polymerase 1 (PARP1) were increased compared with individual drug exposures (Fig. 5A-i). The cleavage of caspase-3 correlated with

![Figure 3](image-url)

**Figure 3.** Activation of the extrinsic pathway plays a central role in the toxicity of flavopiridol and vorinostat as individual agents, and in combination, in breast cancer cells. A, human mammary carcinoma cells (MDA-MB-231) were plated as in Methods and, 24 h after plating, pretreated with a pan-caspase inhibitor (zVAD, 50 µmol/L); a caspase-9 inhibitor (LEHD, 50 µmol/L) or a caspase-8 inhibitor (IETD, 50 µmol/L) followed 30 min later by concurrent treatment with vehicle control (VEH, DMSO) or flavopiridol (FP, 100 nmol/L) or vorinostat (Vor., 500 nmol/L). Viability was determined in triplicate 96 h after drug exposure using trypan blue exclusion assays (± SE, n = 3); *, P < 0.05, reduced level of killing compared with vehicle-treated cell lacking caspase inhibitor. B, human mammary carcinoma cells (MDA-MB-231) were plated as in Methods and, 12 h after plating, were infected with either empty vector control virus (CMV), a virus to express dominant-negative caspase-9, a recombinant virus to express the caspase-8 inhibitor CRM A, or with both viruses. Twenty-four hours after infection, cells were treated with flavopiridol (100 nmol/L) or vorinostat (500 nmol/L). Viability was determined in triplicate 96 h after drug exposure using trypan blue exclusion assays (± SE, n = 3); *, P < 0.05, reduced level of killing compared with CMV-infected cells lacking suppression of caspase function. Inset, MDA-MB-231 cells were transfected with a plasmid to express green fluorescent protein (GFP)–tagged form of LC3 that will vacuolize in autophagic vacuoles. MDA-MB-231 cells expressing LC3-GFP were treated with flavopiridol and vorinostat or vehicle (DMSO), and 24 and 48 h after drug exposure, the cells examined for the presence of autophagic vacuoles (n = 2). C, human mammary carcinoma cells (MCF7) were plated as in Methods and, 24 h after plating, concurrently pretreated with a pan-caspase inhibitor (50 µmol/L), a caspase-9 inhibitor (50 µmol/L) or a caspase-8 inhibitor (50 µmol/L), followed 30 min later by treatment with vehicle control (DMSO) or flavopiridol (100 nmol/L) or vorinostat (500 nmol/L). In parallel studies, 12 h after plating, MCF7 cells were infected with either empty vector control virus (CMV) or a recombinant virus to express the caspase-8 inhibitor CRM A. Twenty-four hours after infection, cells were treated with flavopiridol (100 nmol/L) or vorinostat (500 nmol/L). For small molecule inhibitor or viral infected cells, viability was determined in triplicate 96 h after drug exposure using trypan blue exclusion assays (± SE, n = 3); *, P < 0.05, reduced level of killing compared with vehicle-treated/CMV-infected cells lacking suppression of caspase function.
reduced phosphorylation of ERK1/2, AKT, and p38 MAPK, with little alteration in the phosphorylation of JNK1/2 (Fig. 5A-ii). Treatment of cells with flavopiridol, vorinostat, or the drugs in combination reduced expression of BCL-xL, whereas treatment with flavopiridol reduced expression of c-FLIP proteins. Furthermore, treatment of cells with vorinostat increased both IκB protein levels and IκB S32 phosphorylation, effects that were suppressed by flavopiridol.

To assess the functional significance of these events on breast cancer cell survival, studies were done in which XIAP, c-FLIP-s, or BCL-xL were overexpressed in drug-treated MDA-MB-231 cells. Notably, overexpression of XIAP did not significantly alter drug toxicity, whereas overexpression of c-FLIP-s significantly reduced lethality induced by vorinostat and the vorinostat/flavopiridol regimen. Interestingly, overexpression of BCL-xL reduced the toxicity of all drug treatments to levels below those induced by flavopiridol alone in vector control-infected cells (Fig. 5B). These findings are in agreement with data using caspase inhibition and transformed MEFs lacking expression of proapoptotic proteins and collectively argue that flavopiridol and vorinostat promote cell killing via caspase-8−, cathepsin- and BID-dependent mechanisms.
The activities of both AKT and ERK1/2 were suppressed to varying extents by flavopiridol and vorinostat exposure and expression of dominant-negative AKT enhanced both flavopiridol and vorinostat toxicity. Although flavopiridol suppressed ERK1/2 phosphorylation, expression of constitutively activated MEK1 neither reduced flavopiridol toxicity nor suppressed enhanced toxicity in flavopiridol- and vorinostat-treated cells (Fig. 5C). Expression
of activated AKT did not alter flavopiridol toxicity, but significantly suppressed the potentiation of flavopiridol lethality by vorinostat. Based on these findings, the impact of activated AKT and MEK1 on the expression of caspase-8 and mitochondrial regulatory proteins was examined. Expression of activated MEK1 and activated AKT both reduced the suppression of c-FLIP-s, XIAP, BCL-xL, and MCL-1 protein levels in cells treated with flavopiridol and vorinostat (Fig. 5C, inset). Collectively, these data argue that flavopiridol acts to reduce AKT activity, an effect that is enhanced by vorinostat, leading to reduced c-FLIP protein expression. This leads, in turn, to the promotion of caspase-8–dependent death-related signaling.

In leukemic cells, activation of JNK1/2 and inhibition of NF-κB function have been linked to lethal interactions between flavopiridol and vorinostat (e.g., ref. 27). Treatment of MDA-MB-231 cells with a JNK1/2 inhibitory peptide did not modify the lethal interaction between flavopiridol and vorinostat, whereas expression of dominant-negative IκB enhanced the lethality of flavopiridol and of flavopiridol/vorinostat treatment. However, expression of dominant-negative IκB did not alter the apparent greater than additive lethal interaction between these agents (Fig. 5D). Moreover, expression of dominant-negative AKT, but not dominant-negative MEK1, enhanced the lethality of both flavopiridol and of vorinostat and modified the toxicity of combined drug exposure to an effect that became a simple additive effect (Fig. 5D, data not shown). Collectively, these findings argue that in breast cancer cells, modulation of the phosphoinositide-3-kinase–AKT pathway function may play a greater functional role in flavopiridol and vorinostat lethality than changes in ERK1/2, NF-κB, and JNK1/2 activity.

Discussion

The studies described herein were designed to explore the mechanisms by which the CDK inhibitor flavopiridol and the HDACI vorinostat, administered at relatively low, potentially clinically relevant concentrations, interact to kill mammary carcinoma cells in vitro. These studies have particular relevance because phase I trials of this drug combination have recently been initiated in patients with refractory acute myelogenous leukemia and solid tumor malignancies. Previous studies have shed light on the mechanisms by which HDACIs and flavopiridol interact to induce apoptosis in human leukemia cells, including disruption of p21 induction, down-regulation of MCL-1, and inactivation of NF-κB, among others (e.g., ref. 27 and references therein). However, whether similar interactions occur in epithelial tumors in general, and breast cancer cells in particular, has not been examined.

In a previous study, treatment of breast cancer cells with vorinostat concentrations ≥2.5 μmol/L resulted in profound growth arrest accompanied by evidence of breast cancer cell maturation (28). In another report, exposure of cells to vorinostat concentrations of 2.0 to 5.0 μmol/L resulted in multiple perturbations in the expression of BCL-2 family members that would be expected to promote apoptosis (29). It is noteworthy that in the present study, synergistic, sequence-dependent interactions between flavopiridol and vorinostat were observed at drug concentrations approximately 1 log lower than those reported in earlier investigations. Although plasma vorinostat concentrations in excess of 1 μmol/L have been reported in patients receiving 400 mg p.o. BID, the concentrations employed in the present study are likely to be more readily pharmacologically achievable.

It is noteworthy that flavopiridol lethality in mammary carcinoma cells was weakly suppressed by a pan-caspase inhibitor and was modestly, albeit significantly reduced by loss of cathepsin protease function, whereas vorinostat toxicity was suppressed by pan-caspase inhibition and by inhibition of caspase-8. On the other hand, the marked lethality of the vorinostat/flavopiridol regimen was determined to be caspase-8– and cathepsin B–dependent and required proteolytic processing of the BH3 domain protein BID, which promotes mitochondrial dysfunction and a caspase-9–dependent induction of cell death. Collectively, these observations suggest that the mechanisms underlying potentiation of the lethality of low concentrations of vorinostat by flavopiridol differ significantly from those responsible for breast cancer cell death following exposure to considerably higher vorinostat concentrations administered as a single agent, as well as those responsible for apoptosis induction by the vorinostat/flavopiridol regimen in leukemia cells. Collectively, the present findings argue that in breast cancer cells, activation of the extrinsic apoptotic pathway as well as cathepsin protease-dependent events play important roles in flavopiridol/vorinostat lethality.

Treatment of MDA-MB-231 cells with flavopiridol suppressed c-FLIP-s, and BCL-xL expression and treatment with vorinostat also reduced BCL-xL expression. Notably, combined flavopiridol and vorinostat treatment abolished c-FLIP-s and c-FLIP-l expression and, in addition, decreased MCL-1 levels. These findings are in general agreement with the known roles of these proteins in cell survival, and with cell viability findings arguing that the combination of these agents induced cell killing via both caspase-8– and caspase-9–dependent pathways. Such observations are also in accord with previous studies indicating that combined activation of the intrinsic and extrinsic apoptotic pathways cooperate to promote cell death (30). It is noteworthy that ectopic expression of c-FLIP-s abolished the ability of vorinostat to potentiate flavopiridol toxicity, and ectopic expression of BCL-xL suppressed the lethality of all agents, administered alone or in combination. Such findings argue strongly that down-regulation of these antiapoptotic proteins play a significant role in synergistic interactions between these therapeutic agents.

The mechanisms by which flavopiridol and vorinostat down-regulate the expression of these antiapoptotic proteins may be multifactorial. For example, flavopiridol, by inhibiting the PTEF-b transcription complex, acts as a transcriptional repressor and can block the transcription of
short-lived proteins including XIAP (ref. 22 and references therein). Although HDACIs like vorinostat are generally thought to promote chromatin relaxation and transcription, DNA array analysis has shown that HDACIs down-regulate as many genes as they up-regulate (31). Consequently, it seems plausible that in breast cancer cells, vorinostat and flavopiridol down-regulate the expression of multiple antiapoptotic proteins that cooperate to trigger cell death.

Deletion of BAX and BAK function modestly suppressed flavopiridol toxicity, but abolished the potentiation of vorinostat lethality. Such findings are in accord with previous studies indicating that loss of these multi-domain BCL-2 family members protects cells from diverse noxious stimuli (32). It is also significant that the BH3-only domain protein BID was cleaved following treatment of breast cancer cells and MEFs with flavopiridol and vorinostat, and BID cleavage was catalyzed by both caspase-8 and by cathepsin proteases. There has been some controversy concerning whether certain proapoptotic BH3-only proteins such as BIM act directly to induce mitochondrial injury or instead act to neutralize the actions of multi-domain antiapoptotic proteins such as BCL-2 or BCL-xL. In this context, recent evidence supports the latter model (33). On the other hand, it is generally recognized that BID, or its active, truncated form (tBID) acts directly on mitochondria to trigger release of proapoptotic proteins (i.e., cytochrome c) into the cytosol (34). Death receptor signaling, which comprises the extrinsic apoptotic cascade, involves a pathway that proceeds via caspase-8 signaling, leading to either BID cleavage and mitochondrial dysfunction, or directly to procaspase-3 cleavage, in each case culminating in cell death. Such a model could account for the ability of genetic or pharmacologic interruption of the extrinsic pathway to attenuate flavopiridol/vorinostat lethality. In addition, cathepsin proteases have also been shown to play a significant role in tumor necrosis factor α (TNF-α) and FAS-stimulated cell death processes and are also known to cooperate with caspase-8 to trigger mitochondrial dysfunction and cell death (35, 36). Palacios et al. (37) recently showed that flavopiridol (100 nmol/L) potentiated TRAIL lethality in MDA-MB-231 cells, and that this effect was due to the suppression of c-FLIP-s/1 expression by flavopiridol. It is therefore possible that cooperation between the activation of the extrinsic pathway and cathepsin protease–dependent events contribute to the enhanced lethality of the flavopiridol/vorinostat regimen in breast cancer cells. In this context, it will be of interest to determine whether agents that activate the extrinsic apoptotic pathway also enhance the activity of the flavopiridol/vorinostat regimen in such cells, and if so, whether calpain or cathepsin proteases might be involved in this phenomenon. Studies involving leukemia and lymphoma models suggest that flavopiridol and vorinostat interact in these cells by suppressing NF-κB function, decreasing the expression of mitochondrial protective proteins such as MCL-1, and activating the JNK1/2 pathway (e.g., refs. 17, 20, 22, 27). Such findings are consistent with evidence that flavopiridol acts as an inhibitor of IKK and, by extension, NF-κB activation (18). The present studies suggest that some differences exist between the mechanism(s) of interaction of these agents in breast cancer cells and leukemic cells. For example, in contrast to the case of breast cancer cells, disruption of the extrinsic apoptotic pathway, through inhibition of FADD function, failed to modify flavopiridol/vorinostat lethality in leukemia cells. Furthermore, inhibition of JNK1/2 failed to alter the toxicity of flavopiridol and/or vorinostat in breast cancer cells, in contrast to data in leukemic cells. Moreover, in breast cancer cells, inhibition of NF-κB function by expression of a dominant-negative IκB protein only modestly enhanced the toxicity of flavopiridol with or without vorinostat, whereas lethal potentiating effects of dominant-negative IκB protein expression were considerably greater in the case of leukemia cells. Collectively, these findings indicate that although the signaling events responsible for flavopiridol/vorinostat lethality in breast cancer cells share certain common features with events in human leukemia cells, important differences exist.

In clinical trials using a 72-h infusion schedule, the predicted free plasma concentrations of flavopiridol were found to be ~10% of the total amount of infused drug, with peak free plasma concentrations in the 25–80-nmol/L range (ref. 38 and references therein). These drug levels caused significant toxicities in patients with modest apparent benefit in terms of tumor control. Hence, based on patient performance and tumor response rates, alternate schedules of flavopiridol infusion were explored, with the rate of drug administration being increased in many trials to 1–24 h, achieving similar free flavopiridol concentrations with objective clinical responses being noted (refs. 38, 39, and references therein). More recently, a novel loading and 4-h flavopiridol infusion schedule has been described, which results in higher and more sustained plasma flavopiridol concentrations (40). As noted previously, plasma vorinostat concentrations in excess of 1 μmol/L have been reported (9, 41). Although such concentrations approach those previously shown to induce cell death in breast cancer cells in vitro, it is unlikely that such concentrations can be sustained in patients. It is therefore noteworthy that considerably lower vorinostat concentrations (i.e., 250 nmol/L) resulted in synergistic interactions with flavopiridol in breast cancer cells in vitro, raising the possibility that lethal concentrations of these agents may be achievable in vivo. Based on the present observations, evaluation of regimens combining flavopiridol and vorinostat in breast cancer deserve consideration. Accordingly, phase I trials of flavopiridol and vorinostatin in patients with refractory solid tumor malignancies are currently under way.

References


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Extrinsic pathway- and cathepsin-dependent induction of mitochondrial dysfunction are essential for synergistic flavopiridol and vorinostat lethality in breast cancer cells

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