Vorinostat and Sorafenib Synergistically Kill Tumor Cells via FLIP Suppression and CD95 Activation


Abstract

Purpose and Design: Mechanism(s) by which the multikinase inhibitor sorafenib and the histone deacetylase inhibitor vorinostat interact to kill hepatic, renal, and pancreatic adenocarcinoma cells has been defined.

Results: Low doses of sorafenib and vorinostat interacted in vitro in a synergistic fashion to kill hepatic, renal, and pancreatic adenocarcinoma cells in multiple short-term viability (24–96 h) and in long-term colony formation assays. Cell killing was suppressed by inhibition of cathepsin proteases and caspase-8 and, to a lesser extent, by inhibition of caspase-9. Twenty-four hours after exposure, the activities of extracellular signal-regulated kinase 1/2, AKT, and nuclear factor-κB were only modestly modulated by sorafenib and vorinostat treatment. However, 24 h after exposure, sorafenib- and vorinostat-treated cells exhibited markedly diminished expression of c-FLIP-s, full-length BID, BCL-2, BCL-XL, MCL-1, XIAP, increased expression of BIM, and increased activation of BAX, BAK, and BAD. Expression of eIF2α S51A blocked sorafenib- and vorinostat-induced suppression of c-FLIP-s levels and overexpression of c-FLIP-s abolished lethality. Sorafenib and vorinostat treatment increased surface levels of CD95 and CD95 association with caspase-8. Knockdown of CD95 or FADD expression significantly reduced sorafenib/vorinostat-mediated lethality.

Conclusions: These data show that combined exposure of epithelial tumor cell types to sorafenib and vorinostat diminishes expression of multiple antianpoptotic proteins and promotes activation of the CD95 extrinsic apoptotic and the lysosomal protease pathways, and that suppression of c-FLIP-s expression represents a critical event in transduction of the proapoptotic signals from CD95 to promote mitochondrial dysfunction and death.

In the United States, hepatoma is diagnosed in ~19,000 patients per annum with ~17,000 deaths from the disease, with a 5-year survival rate of <10%. Hepatoma is a leading cause of diagnosed cancer in Africa and Asia and represents the fifth most commonly diagnosed malignancy in the world (1, 2). In the United States, pancreatic and renal cancer are diagnosed in ~37,000 and ~31,000 patients per annum, respectively, with ~34,000 and ~13,000 deaths from each disease every year (1, 2). Pancreatic and renal cancers have 5-year survival rates of <5% and 5% to 10%, respectively. These statistics emphasize the need to develop novel therapies against these lethal malignancies.

The Raf/mitogen-activated protein kinase (MAPK) kinase 1/2 (MEK1/2)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway is frequently dysregulated in neoplastic transformation (3–5). The MEK1/2-ERK1/2 module comprises, along with c-Jun NH2-terminal kinase (JNK1/2) and p38 MAPK, members of the MAPK superfamily. These kinases are involved in responses to diverse mitogens and environmental stresses, including DNA damage, osmotic stress, and hypoxia, and have also been implicated in multiple cellular functions, including proliferation, differentiation, and cell survival processes. Although exceptions exist, activation of the ERK1/2 pathway is generally associated with cell survival, whereas induction of JNK1/2 and p38 MAPK pathways generally signals apoptosis. There is also evidence that the net balance of signals related to the amplitude and duration of the cytoprotective ERK1/2 and the stress-related JNK1/2 and p38 MAPK pathways determines whether a cell lives or dies following various insults (3–5). Although the mechanism(s) by which ERK1/2 activation promotes survival is not known with certainty, several
downstream antiapoptotic effector proteins have been identified, including direct inhibition of proapoptotic proteins, such as caspase-9, BAD, and BIM, and increased expression of antiapoptotic proteins, such as BCL-XL, MCL-1, and c-FLIP (6–11). In view of the importance of the Raf-MEK1/2-ERK1/2 pathway in neoplastic cell survival, clinically relevant inhibitors have been developed and have now entered clinical trials, including sorafenib (Bay 43-9006, Nexavar; a Raf kinase inhibitor) and AZD6244 (a MEK1/2 inhibitor; refs. 12, 13).

Sorafenib is a multi kinase inhibitor that was originally developed as an inhibitor of Raf-1 but which was subsequently shown to inhibit multiple other kinases, including platelet-derived growth factor, vascular endothelial growth factor receptors 1 and 2, c-Kit, and FLT3 (14). Antitumor effects of sorafenib in renal cell carcinoma and in hepatoma have been ascribed to antiangiogenic actions of this agent through inhibition of multiple growth factor receptors (15–17). However, several groups, including our own, have shown in vitro that sorafenib kills human leukemia cells at concentrations below those achievable in the plasma (e.g., a C\text{max} of 15-20 \mu M/L) through a mechanism involving down-regulation of the antiapoptotic BCL-2 family member MCL-1 (18, 19). In these studies, sorafenib-mediated MCL-1 down-regulation occurred through a translational rather than a transcriptional or posttranslational process that was mediated by endoplasmic reticulum (ER) stress signaling (20, 21). This suggests that the previously observed antitumor effects of sorafenib are partially mediated by a complex combination of inhibition of Raf family kinases and the ERK1/2 pathway, receptor tyrosine kinases that signal angiogenesis, and the induction of ER stress signaling.

Histone deacetylase inhibitors (HDACi) represent a class of agents that act by blocking histone deacetylation, thereby modifying chromatin structure and gene transcription. HDACs, along with histone acetyltransferases, reciprocally regulate the acetylation status of the positively charged NH\text{2}-terminal histone tails of nucleosomes. In general, HDACs promote histone acetylation and neutralization of positively charged lysine residues on histone tails, allowing chromatin to assume a more relaxed, open conformation, which favors gene transcription (22). However, HDACs also induce acetylation of many other nonhistone targets, actions that may have pleiotropic biological consequences, including inhibition of HSP90 function, induction of oxidative injury, disruption of checkpoint control, and up-regulation of death receptor expression (23–25). With respect to combinatorial drug studies with a multi kinase inhibitor such as sorafenib, HDACs are of particular interest in that they also down-regulate/inactivate multiple oncogenic kinases by interfering with HSP90 function, leading to proteosomal degradation of these proteins. Vorinostat (suberoylanilide hydroxamic acid, Zolinza) is a hydroxamic acid HDACi that has shown preliminary preclinical evidence of activity in hepatoma and other malignancies with a C\text{max} of \sim 9 \mu M/L (26–28). Based on the single-agent activity of sorafenib in hepatoma patients, as well as its approval for treatment of patients with renal cell carcinoma and hepatocellular carcinoma, and the fact that sorafenib and vorinostat target multiple overlapping pathways implicated in tumor cell survival, the possibility arose that a combined approach might be more effective than either agent administered individually. The present studies reveal that sorafenib and vorinostat interact in a highly synergistic manner to induce cell death in hepatoma, pancreatic, and renal cell carcinoma cells through multiple interacting mechanisms, most notably enhanced activation of the extrinsic apoptotic pathway.

**Materials and Methods**

**Materials**

Sorafenib and sorafenib tosylate (Bay 43-9006 and BAY 54-9085; Bayer) as well as vorinostat (Merck) were provided by the Cancer Treatment and Evaluation Program, National Cancer Institute/NIH (Bethesda, MD). Phosphorylated (total) (ERK1/2, JNK1/2, and p38 MAPK) antibodies, phosphorylated (total) AKT (T308; S473), and the total and cleaved caspase-3 antibodies were purchased from Cell Signaling Technology. Anti-BID and anti-cathepsin B antibodies were purchased from Cell Signaling Technology. All the secondary antibodies (anti-rabbit horseradish peroxidase, anti-mouse horseradish peroxidase, and anti-goat horseradish peroxidase) were purchased from Santa Cruz Biotechnology. Enhanced chemiluminescence and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) kits were purchased from NEN Life Science Products and Boehringer Mannheim, respectively. Trypsin-EDTA, DMEM, RPMI 1640, and penicillin-streptomycin were purchased from Life Technologies. HEPG2, Hep3B, HuH7, A498, CAKI-1, MiaPaCa2, and Panc1 cells were purchased from the American Type Culture Collection. UOK121LN cells were kindly provided by Dr. Lineham Marston (NIH). BAK\textsuperscript{–/–}, BAK\textsuperscript{−/−}, and BID\textsuperscript{−/−} fibroblasts were kindly provided by Dr. S. Korsmeyer (Harvard University, Boston, MA). Commercially available validated short hairpin RNA molecules to knock down RNA/protein levels were from Qiagen; CD95 (S102654463; S10318255) and FADD (S100300223; S103648911). Reagents and performance of experimental procedures were in general as described in refs. 20, 21, 29–33.

**Methods**

**Culture and in vitro exposure of cells to drugs.** All established cell lines were cultured at 37°C [5% (v/v) CO\textsubscript{2}] in vitro using RPMI 1640 supplemented with 5% (v/v) FCS and 10% (v/v) nonessential amino acids. For short-term cell killing assays, immunoblotting, and apoptosis-inducing factor/cathepsin release studies, cells were plated at a density of 3 \times 10\textsuperscript{5}/cm\textsuperscript{2} (2–2 \times 10\textsuperscript{5} per well of a 12-well plate) and, 48 h after plating, treated with various drugs. Unless otherwise indicated, cells were plated in triplicate and treated with vehicle (DMSO), sorafenib (3.0 \mu M/L), vorinostat (500 nmol/L), or both sorafenib and vorinostat. In vitro vorinostat and sorafenib treatments were from 100 nmol/L stock solutions of each drug and the maximal concentration of vehicle (DMSO) in medium was 0.02% (v/v). Cells were not cultured in reduced serum medium during any study in this article.

**In vitro cell treatments, microscopy, SDS-PAGE, and Western blot analysis.** For in vitro analyses of short-term cell death effects, cells were treated with vehicle or vorinostat/sorafenib for the indicated times in the figure legends. For apoptosis assays where indicated, cells were pretreated with vehicle (DMSO), ZVAD (50 \mu M/L), calpain inhibitor [acetyl-calpastatin (amino acids 184-210); 5 \mu M/L], or cathepsin B inhibitor ([L-3-(propylcarbamoyl)oxirane-2-carbonyl]-i-isoueryl-i-proline methyl ester; 1 \mu M/L]; isolated at the indicated times; either subjected to trypan blue cell viability assay by counting in a light microscope or fixed to slides; and stained using a commercially available Diff-Quick (Giemsa) assay kit. Alternatively, the Annexin V/propidium iodide assay was carried to determine cell viability out as per the manufacturer’s instructions (BD Pharmingen) using a Becton Dickinson FACScan flow cytometer. Vorinostat/sorafenib lethality, as judged by Annexin V/propidium iodide, was first evident \sim 24 h after drug exposure (data not shown).

For immunohistochemistry of cells fixed in situ, fixed cells were blocked 1 h with antibody dilution buffer 2% (v/v) rat serum, 1%...
[w/v] bovine serum albumin in Dulbecco’s PBS] and then incubated overnight at 4°C in antibody dilution buffer with addition of anti-CD95 antigen (2 µg/mL; Abcam). Cells were then washed and incubated for 1 h with a 488 nm–tagged secondary antibody (Invitrogen). Cells were washed, coverslipped, and analyzed on a fluorescent microscope (×100 magnification).

For SDS-PAGE and immunoblotting, cells were plated at 5 × 10⁵/cm², treated with drugs at the indicated concentrations and after the indicated time of treatment, and lysed in whole-cell lysis buffer [0.5 mol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.02% bromphenol blue], and the samples were boiled for 30 min. 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 enhanced chemiluminescence.

For presentation, immunoblots were digitally scanned at 600 dpi using Adobe Photoshop CS2, and their color was removed and figures were generated in Microsoft PowerPoint.

**Infection of cells with recombinant adenoviruses.** Cells were plated at 3 × 10⁴/cm² in each well of a 12-well, 6-well, or 60-mm plate. After plating (24 h), cells were infected (hepatoma and pancreatic carcinoma; at a multiplicity of infection of 50; UOK1211LN renal carcinoma at a multiplicity of infection of 400) with a control empty vector virus [cytomegalovirus (CMV)] and adenoviruses to express ERmA, c-FLIP-s, BCL-XL, and XIAP or to express dominant-negative AKT/MEK1/II

**Transfection of cells with small interfering RNA or with plasmids.** For plasmids: Cells were plated as described above and, 24 h after plating, transfected with a variety of constructs. For mouse embryonic fibroblasts (MEF; 2-5 µg) or other cell types (0.5 µg), plasmids expressing a specific mRNA [or small interfering RNA (siRNA)] or appropriate vector control plasmid DNA was diluted in 50 µL serum-free and antibiotic-free medium (one portion for each sample). Concurrently, 2 µL Lipofectamine 2000 (Invitrogen) was diluted into 50 µL of serum-free and antibiotic-free medium (one portion for each sample). Diluted DNA was added to the diluted Lipofectamine 2000 for each sample and incubated at room temperature for 30 min. This mixture was added to each well/dish of cells containing 200 µL serum-free and antibiotic-free medium for a total volume of 300 µL, and the cells were incubated for 4 h at 37°C. An equal volume of 2× medium was then added to each well. Cells were incubated for 48 h, then treated with vorinostat/sorafenib, and subsequently analyzed. (b) For nuclear factor-kB (NF-kB) promoter-luciferase assays: Cells or MEFs were plated as described above and, 24 h after plating, transfected with either a control luciferase plasmid (1 µg) + β-galactosidase plasmid (15 ng) or luciferase plasmid with 4× nuclear factor-kB consensus binding sequences (1 µg) + β-galactosidase plasmid (15 ng), incubated for 5 min in serum-free medium, then added to Genejuice (2 µL per condition; EMD Biosciences), and incubated for 15 min together at room temperature. This mixture was added to cells and incubated at 37°C for 24 h, after which cells were treated with vorinostat/sorafenib for 0 to 24 h, then washed twice with PBS, and harvested in cell lysis buffer [25 mmol/L Tris phosphate (pH 7.8), 2 mmol/L DTT, 2 mmol/L CDTA, 10% glycerol, 1% (v/v) Triton X-100]. The lysate was centrifuged for 5 min at 15,000 × g at 4°C to pellet debris. The luciferase assay was done according to the manufacturer’s instructions (Promega). Briefly, luciferase substrate was brought to room temperature, then added to 20 µL lysate, and measured immediately on a Perkin-Elmer luminometer. The luciferase measurement was normalized to β-galactosidase measurement to control for transfection efficiency; 50 µL of 2× β-galactosidase reagent [200 mmol/L NaH₂PO₄, NaH₂PO₄, (pH 7.4), 2 mmol/L MgCl₂, 200 mmol/L β-mercaptoethanol, 1.34 mg/mL O-nitrophenyl-β-D-galactopyranosidase] were added to 50 µL cell lystate and incubated at 37°C for 10 min. The product of the assay was measured at an absorbance of 405 nm.

**Isolation of a crude cytosolic fraction.** A crude membrane fraction was prepared from treated cells. Briefly, cells were washed twice in ice-cold isotonic HEPES buffer [10 mmol/L HEPES (pH 7.5), 200 mmol/L mannitol, 70 mmol/L sucrose, 1 µmol/L EDTA, 10 µmol/L protease inhibitor cocktail (Sigma)]. Cells on ice were scraped into isotonic HEPES buffer and lysed by passing 20 times through a 25-gauge needle. Large membrane pieces, organelles, and unlysed cells were removed from the suspension by centrifugation for 5 min at 120 × g. The crude granular fraction and cytosolic fraction were obtained by centrifugation for 30 min at 10,000 × g, leaving the cytosol as supernatant.

**In vivo exposure of carcinoma tumors to drugs.** Athymic female NCr-nu/nu mice were obtained from The Jackson Laboratory. Mice were maintained under pathogen-free conditions in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture (Washington, DC), the U.S. Department of Health and Human Services (Washington, DC), and the NIH. HEP3B cells were cultured and isolated by trypsinization followed by cell number determination using a hemacytometer. Cells were resuspended in PBS and 10 million tumor cells per 100 µL PBS were injected into the right rear flank of each mouse, and tumors were permitted for form to reach a volume of ~150 mm³ over the following 6 wk. The tumor take rate for HEP3B tumors was ~20%. Vials of vorinostat (stored in a -20°C cold room under vacuum and protected from light) were first dissolved in 30 µL DMSO, diluted in sterile saline, and heated to boiling for complete dissolution before injection. Mice were administered 25 mg/kg vorinostat by oral gavage based on body mass (0.2 mL total volume per 30 g mouse). Animals received two more administrations of vorinostat, 24 h apart, for an additional 2 d. Sorafenib was administered 30 min before the first vorinostat administration each day. Sorafenib tosylate (BAY 54-9085) was dissolved fresh each day. The dosing volume used was 0.3 mL/30 g body weight. The compound was dissolved in a 50% Cremophor EL/50% ethanol mixture. The mixture was heated to 60°C and sonicated for 20 min to solubilize. Once the BAY 54-9085 entered solution, the aqueous component was added gradually and with mixing to generate the 1× dosing solution. Animals were administered with BAY 54-9085 for a final concentration of 45 mg/kg. Animals received two more administrations of vorinostat, 24 h apart, for an additional 2 d. Each animal not receiving a dose of sorafenib or vorinostat at the time of drug treatment was administered diluent alone in a volume equal to the amount given with the drug.

**Ex vivo manipulation of tumors.** Animals were euthanized by CO₂ and placed in a BL2 cell culture hood on a sterile barrier mat. The
Fig. 1. Sorafenib and vorinostat interact in a synergistic fashion to kill pancreatic, liver, and kidney tumor cells in colony formation assays. A, pancreatic (PANC-1) and hepatoma (HEP3B) cells were plated as single cells (250-1,500 per well) in sextuplicate and, 12 h after plating, treated with vehicle (VEH; DMSO), sorafenib (Sor; 3.0-6.0 μmol/L), or vorinostat (Vor; 250-500 nmol/L) or with both drugs combined, as indicated at a fixed concentration ratio to do median dose effect analyses for the determination of synergy. After drug exposure (48 h), the medium was changed and cells were cultured in drug-free medium for an additional 10 to 14 d. Cells were fixed and stained with crystal violet, and colonies of >50 cells/colony were counted. Columns, true percentage inhibition of colony formation plotted from the means of sextuplicate plates from two separate experiments (n = 3 total studies); bars, SE. *, P < 0.05, greater cell killing than compared with any other treatment condition. Colony formation data were also entered into the CalcuSyn program and CI values were determined. A CI value of 1.00 indicates synergy: CI values for PANC-1 and HEP3B were all below 0.70. B, HEPG2 cells were plated in triplicate and treated with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. After drug exposure (48 h), cells were isolated, treated with Annexin V and propidium iodide according to the manufacturer’s instructions, and stained using these established methods. Cells were subjected to flow cytometry to determine the numbers of Annexin V–positive and propidium iodide–positive cells ± SE. Data shown are from a representative of three independent studies. *, P < 0.05, greater cell killing than compared with any other treatment condition. C, UOK121LN and HEPG2 cells were plated in triplicate and treated with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. After drug exposure (48 and 96 h), cells were isolated and stained using trypan blue indicative of later stages of cell death/plasma membrane disruption as described in Materials and Methods. Columns, percentage of trypan blue–positive cells; bars, SE. Data are from a representative of three independent studies. *, P < 0.05, greater cell killing than compared with any other treatment condition.
bodies of the mice were soaked with 70% (v/v) ethanol and the skin around the tumor was removed using small scissors, forceps, and a disposable scalpel. These implements were flame sterilized between removal of the outer and inner layers of skin. A piece of the tumor (~50% by volume) was removed and placed in a 10-cm dish containing 5 mL of RPMI 1640 cell culture medium on ice. In parallel, the remainder of the tumor was placed in 5 mL of Streck tissue fixative (Fisher Scientific) in a 50 mL conical tube for H&E fixation. The tumor sample that had been placed in RPMI 1640 was minced with a sterile disposable scalpel into the smallest possible pieces and then placed in a sterile disposable flask. The dish was rinsed with 6.5 mL of RPMI 1640, which was then added to the flask. A 10× solution of collagenase (2.5 mL, 28 units/mL final concentration; Sigma) and 10× of enzyme mixture containing DNase (308 units/mL final concentration; Sigma) and Pronase (22,500 units/mL final concentration; EMD Sciences) in a volume of 1 mL was added to the flask. The flasks were placed into an orbital shaking incubator at 37 °C for 1.5 h at 150 rpm. Following digestion, the solution was passed through a 0.4-μm filter into a 50 mL conical tube. After mixing, a sample was removed for viable and total cell counting using a hemacytometer. Cells were centrifuged at 500 × g for 4 min, the supernatant was removed, and fresh RPMI 1640 containing 10% (v/v) FCS was added to give a final resuspended cell concentration of 1 × 10^6 cells/mL. Cells were diluted and plated in 10-cm dishes in triplicate at a concentration of 2 × 10^3 to 6 × 10^3 per dish for control, sorafenib, and vorinostat treatments and 4 × 10^3 to 10 × 10^3 per dish for combined sorafenib and vorinostat exposure.

**Immunohistochemistry and staining of fixed tumor sections.** After sacrifice, tumors were fixed in OCT compound (Tissue-Tek) and cryostat sectioned (Leica) as 12-μm sections. Nonspecific binding was blocked with a 2% (v/v) rat sera, 1% (v/v) bovine sera, 0.1% (v/v) Triton X-100, and 0.05% (v/v) Tween 20 solution and then sections were stained for cell signaling pathway markers: cleaved caspase-3 (rabbit IgG, 1:100; Cell Signaling Technology), phosphorylated ERK1/2/3 (S473; mouse IgG, 1:100; Santa Cruz Biotechnology), and phosphorlated AKT1/2/3 (S473; mouse IgG, 1:100; Santa Cruz Biotechnology), and phosphorlated ERK1/2 (mouse IgG, 1:100; Santa Cruz Biotechnology). For staining of sectioned tumors, primary antibodies were applied overnight, sections were washed with phosphate buffer solution, and secondary antibodies were applied for detection [as indicated in the figure; goat anti-rat Alexa Fluor 488/647 (1:500; Invitrogen) and goat anti-mouse Alexa Fluor 488/647 (1:500; Invitrogen) secondary antibody as per the primary antibody used] or detected by way of 3,3’-diaminobenzidine substrate peroxidase detection kit (Biogenex) as per the manufacturer’s instructions. Sections were then dehydrated, cleared, and mounted with coverslips using 4’,6-diamidino-2-phenylindole mounting medium (Vectastain). Apoptotic cells with double-stranded DNA breaks were detected using the Upstate TUNEL Apoptotic Detection kit according to the manufacturer’s instructions. Slides were applied to high-powered light/confocal microscopes (Zeiss LSM 510 Meta confocal scanning microscope; Zeiss HBO 100 microscope with AxioCam MRm camera) at the indicated magnification in the figure/figure legend. The proliferation zone that included both tumor and normal tissue was usually selected as the site of interest within 2 mm of, or juxtaposed to, the leading edge of the tumor.

**Data analysis.** Comparison of the effects of various treatments was done using ANOVA and the 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 (±SE). Median dose effect isobologram analyses to determine synergism of drug interaction were done according to the methods of Chou and Talalay using the CalcuSyn program for Windows (Biosoft). Cells are treated with agents at a fixed concentration dose. A combination index (CI) value of 1.00 indicates synergy of interaction between the drugs, a value of 1.00 indicates additivity, and a value of >1.00 equates to antagonism of action between the agents.

**Results**

Treatment of human liver, pancreatic, and kidney tumor cell lines with increasing low relatively concentrations of vorinostat and sorafenib at a fixed dose ratio resulted in a synergistic enhancement in tumor cell killing as measured by median dose effect long-term colony formation assays (Fig. 1A;
Reexpression of the VHL protein in 786 renal carcinoma cells did not significantly alter the synergistic interaction of vorinostat and sorafenib (data not shown). Additional studies, using a variety of short-term cell killing assays, examined the interaction between vorinostat and sorafenib. In HEPG2 and UOK121LN cells, TUNEL, Annexin V-propidium iodide, and trypan blue exclusion viability assays generated quantitatively and qualitatively similar data.

**Fig. 3.** Sorafenib and vorinostat treatment modulates the expression of c-FLIP-s, BCL-XL, and MCL-1 in tumor cells. A, HEPG2 cells were plated and treated 24 h after plating with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. Six and 24 h after drug exposure, cells were isolated and subjected to SDS-PAGE followed by immunoblotting to determine the expression of Bid, procaspase-8, procaspase-3, XIAP, BCL-2, BCL-XL, MCL-1, c-FLIP-s, phosphorylated eIF2α S51 (P-eIF2α S51), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and ERK2. Data are from a representative study (n = 3). B, HEPG2 cells infected to express empty vector (CMV), BCL-XL, XIAP, or c-FLIP-s. Cells were treated with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. Ninety-six hours after drug exposure, cells were isolated and viability was determined by trypan blue assay. Columns, percentage of trypan blue-positive cells; bars, SE. Data are from the mean of three independent studies. *P < 0.05, less cell killing than compared with parallel condition in vehicle-treated cells. C, HEPG2 and UOK121LN cells were plated and treated 24 h after plating with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. Six and 24 h after drug exposure, cells were isolated and subjected to immunoprecipitation and/or SDS-PAGE followed by immunoblotting to determine the expression of BIM, procaspase-8, BAD S112 phosphorylation, BAX activation, BAK activation, and glyceraldehyde-3-phosphate dehydrogenase. Data are from a representative study (n = 3).
In HEPG2 and MiaPaCa2 cells, pan-inhibition of caspase function using zVAD significantly reduced vorinostat and sorafenib lethality, as did inhibition of cathepsin protease function; in UOK121LN cells, the relative role of caspases in the death process seemed to be greater than that of cathepsins (Supplementary Figs. S5-S7). Inhibition of caspase-8 function (e.g., by ectopic expression of CrmA) significantly reduced, to a greater extent than inhibition of caspase-9 (e.g., by ectopic expression of dominant-negative caspase-9), the lethality of vorinostat and sorafenib in our panel of tumor cells (Fig. 2A; Supplementary Figs. S5-S7). Similar cell killing data after vorinostat and sorafenib treatment were obtained using the caspase-8 and caspase-9 inhibitors IETD and LEHD, respectively (data not shown). Based on data showing that inhibition of caspase-8 and caspase-9 significantly reduced vorinostat and sorafenib lethality, genetically modified transformed MEFs lacking expression of various cell survival modulator genes were used. Combined loss of BAX and BAK function or loss of BID function significantly reduced vorinostat and sorafenib lethality in transformed fibroblasts (Fig. 2B). These findings suggest that lysosomal dysfunction plays a role in the killing process and that caspase-8 signaling and the extrinsic pathway are also involved in vorinostat- and sorafenib-induced transformed cell lethality.

To further define the processes of cell death, immunoblotting analyses in vorinostat- and sorafenib-treated HEPG2 and UOK121LN cells were done (Fig. 3; Supplementary Fig. S8). In UOK121LN cells, within 6 h of combined, but not individual, drug exposure, the expression of the proforms of BID, caspase-8, and caspase-3 (Supplementary Fig. S8) declined relative to vehicle control–treated cells. These observations correlated with decreased expression of BCL-2 and c-FLIP-s (Supplementary Fig. S8). The cleaved form of BID was poorly visualized by immunoblotting in all studies using this drug combination (data not shown). Twenty-four hours after drug exposure, the expression of BCL-2, BCL-XL, MCL-1, c-FLIP-s, BID, procaspase-8, and procaspase-3 had further declined after combined, but not individual, drug exposure.

In HEPG2 cells, little obvious change in the expression of any protein was observed 6 h after combined drug exposure with the exception of c-FLIP-s and procaspase-3, whereas 24 h after combined, but not individual, vorinostat and sorafenib exposure, expression of BID, procaspase-8, procaspase-3, XIAP, BCL-2, BCL-XL, MCL-1, and c-FLIP-s was all reduced (Fig. 3A). In both HEPG2 and UOK121LN cells, decreased expression of prosurvival proteins correlated with increased phosphorylation of eIF2α S51; increased phosphorylation of eIF2α S51 is known to correlate with increased activity of this protein and with suppression of translation/initiation in cells (Fig. 3A; ref. 21). Similar immunoblotting data to that obtained in HEPG2 and UOK121LN cells in Fig. 3A was obtained in MiaPaCa2 cells (Supplementary Fig. S9). Of note, cell viability data obtained 24 h after drug exposure argued that drug-treated tumor cells had just begun to display signs of cell death at this interval (e.g., time course data in Fig. 1; data not shown). Thus, the observed reductions in prosurvival protein expression occurred before significant manifestations of cell killing.

The ability of overexpression of proteins whose levels were reduced in Fig. 3A and in Supplementary Figs. S8 and S9 to prevent cell killing was then tested. Overexpression of BCL-XL, XIAP, or c-FLIP-s significantly reduced vorinostat and sorafenib lethality (Fig. 3B; Supplementary Fig. S10). Whether the expression and/or activity of additional proapoptotic proteins correlated with increased cell killing was also determined. Treatment of cells with vorinostat and sorafenib increased the expression of BIM, including promotion of BIM dephosphorylation as well as the dephosphorylation of BAD S112 and the activation of BAX and BAK (Fig. 3C). Based on data showing that c-FLIP-s overexpression largely abolished low-dose vorinostat and sorafenib lethality, regardless of the fact that in parallel drug exposure also suppressed expression of multiple downstream prosurvival proteins and activated BAX and BAK, the possibility that vorinostat- and sorafenib-induced killing was death receptor dependent, specifically CD95 (FAS receptor) dependent, was explored.

Notably, knockdown of FADD or CD95 expression significantly reduced the lethality of low-dose combined vorinostat and sorafenib exposure in HEPG2 and UOK121LN cells (Fig. 4A; Supplementary Fig. S11). Vorinostat and sorafenib exposure in HEPG2 and UOK121LN cells, in a cell type–dependent fashion, also enhanced expression of FAS-L and/or CD95 proteins as well (Supplementary Fig. S12). In addition, combined treatment of HEPG2 and UOK121LN cells with vorinostat and sorafenib promoted the rapid association of procaspase-8 with CD95 (i.e., DISC complex formation; Fig. 4C). Knockdown of CD95 abolished drug-induced procaspase-8 and BID cleavage in total cell lysates (data not shown). Studies in primary hepatocytes treated with death-inducing natural compounds that act via CD95, such as toxic bile acids, have shown that these agents cause plasma membrane localization and clustering of CD95 as part of the receptor activation/hepatocyte killing process (34, 35). Treatment of HEPG2 cells with vorinostat and sorafenib caused increased plasma membrane localization of CD95 and the appearance of intense-staining punctate bodies containing CD95, demonstrative of CD95 clustering and its activation (Fig. 4D). Note that in HEPG2 cells, CD95 activation and DISC formation occurred at time points (i.e., 6 h) without alteration in either total protein levels of CD95 or FAS-L/FAS-L cleavage, arguing that CD95 activation was ligand independent. Overexpression of c-FLIP-s significantly suppressed vorinostat and sorafenib lethality as measured in TUNEL assays and markedly diminished cytochrome c release into the cytosol of HEPG2 cells (Supplementary Fig. S13).

We next defined how combined exposure to vorinostat and sorafenib could rapidly suppress the expression of multiple prosurvival proteins. High doses of sorafenib, >3 μmol/L as a single agent, have been shown by our laboratories to cause ER stress, translational inhibition, and reduced expression of MCL-1 that correlated with eIF2α phosphorylation (19, 21); in the present studies, lower doses of sorafenib (~3 μmol/L) do not enhance eIF2α phosphorylation but did synergize with vorinostat to cause eIF2α phosphorylation. Expression of dominant-negative eIF2α S51A abolished low-dose combined...
Fig. 4. Sorafenib and vorinostat interact to kill tumor cells via activation of CD95 and suppression of c-FLIP's expression. A, HEPG2 cells were transfected with a nonspecific scrambled control (siSCR) siRNA molecule, or molecules to knock down the expression of CD95 or FADD, according to the manufacturer's instructions. Cells were treated 24 h after transfection with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. Ninety-six hours after drug exposure, cells were isolated and viability was determined by trypan blue assay. Columns, percentage of trypan blue-positive cells (n = 3); bars, SE. #, P < 0.05, less cell killing than compared with parallel condition in vehicle-treated cells.

B, HEPG2 cells were plated and treated 24 h after plating with vehicle or with sorafenib and vorinostat. Cells were isolated at the indicated time points after sorafenib and vorinostat exposure and CD95 was immunoprecipitated from the cell lysate. SDS-PAGE followed by immunoblotting of CD95 immunoprecipitates was done to determine the association of procaspase-8 and caspase-8 with CD95 (n = 3).

C, HEPG2 and UOK121LN cells were plated on glass slides and treated 24 h after plating with vehicle (DMSO) or with sorafenib and vorinostat. Six hours after drug exposure, cells were fixed in situ. Fixed cells were blocked, incubated overnight with anti-CD95 antibody, and then incubated with a 488 nm–tagged fluorescent secondary antibody. Cells were analyzed on a fluorescent microscope. Magnification, ×100. The intensity of CD95 staining was determined at 50 random points per cell for a total of five cells ± SE (n = 3 separate studies).
sorafenib- and vorinostat-induced suppression of c-FLIP-s and MCL-1 expression in HEP3B cells and expression of eIF2α S51A in transformed fibroblasts and in HEP3B cells suppressed the toxic interaction between sorafenib and vorinostat (Fig. 4D; Supplementary Fig. S14). Overexpression of c-FLIP-s in hepatoma and pancreatic cancer cells suppressed the synergistic lethality of vorinostat and sorafenib in median dose effect colony formation assays, reducing the CI value from ~0.45 to 

Table 1. The synergy of killing by sorafenib and vorinostat is dependent on loss of c-FLIP-s expression

<table>
<thead>
<tr>
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<th>MiaPaca2: CMV</th>
<th>Hep3B: CMV</th>
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<tbody>
<tr>
<td><strong>MiaPaca2: CMV</strong></td>
<td>Sorafenib (μmol/L)</td>
<td>Vorinostat (μmol/L)</td>
</tr>
<tr>
<td>3.00</td>
<td>0.250</td>
<td>0.34</td>
</tr>
<tr>
<td>4.50</td>
<td>0.375</td>
<td>0.42</td>
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<tr>
<td>6.00</td>
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| **MiaPaca2: c-FLIP-s** | Sorafenib (μmol/L) | Vorinostat (μmol/L) | Fraction affected | CI | Sorafenib (μmol/L) | Vorinostat (μmol/L) | Fraction affected | CI |
| 3.00     | 0.250         | 0.16       | 0.98          |    | 3.00     | 0.250         | 0.26          | 1.00          |
| 4.50     | 0.375         | 0.21       | 1.23          |    | 4.50     | 0.375         | 0.40          | 1.10          |
| 6.00     | 0.500         | 0.26       | 1.42          |    | 6.00     | 0.500         | 0.54          | 1.08          |

NOTE: Pancreatic (MiaPaca2) and hepatoma (HEP3B) cells were infected to express empty vector (CMV) or c-FLIP-s. Twenty-four hours after infection, cells were plated as single cells (250-1,500 per well) in sextuplicate and, 12 h after plating, treated with vehicle (DMSO), sorafenib (3.0-6.0 μmol/L), or vorinostat (250-500 nmol/L) or with both drugs combined, as indicated at a fixed concentration ratio to do median dose effect analyses for the determination of synergy. Forty-eight hours after drug exposure, the medium was changed and cells were cultured in drug-free medium for an additional 10 to 14 d. Cells were fixed and stained with crystal violet, and colonies of >50 cells/colony were counted. Colony formation data were also entered into the CalcuSyn program and CI and fraction affected values were determined. A CI value of <0.90 to 1.00 indicates synergy, a CI value of 0.90 to 1.10 approximates to additive interactions between the drugs, and a CI value of >1.10 indicates antagonism (n = 2 independent studies).
Fig. 5. Delayed inactivation of ERK1/2 and AKT correlates with profound and long-term loss of c-FLIP expression: delayed activation of NF-κB is a toxic signal following sorafenib and vorinostat exposure. **A**, HEPG2 cells, 24 h after plating, were treated with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. Cells were isolated 24 to 96 h after drug exposure and subjected to SDS-PAGE and immunoblotting to determine the phosphorylation of ERK1/2, JNK1/2, p38 MAPK, and AKT (S473) as well as total ERK2. A representative of three separate studies is shown. **B,** top, HEPG2 cells, 24 h after plating, were infected with empty vector control virus (CMV) or viruses to express constitutively active AKT (caAKT) and constitutively active MEK1 EE (caMEK1 EE). Twenty-four hours after infection, cells were treated with vehicle or with sorafenib and vorinostat. Forty-eight hours after drug exposure, cells were isolated and subjected to SDS-PAGE and immunoblotting to determine the expression of c-FLIP as well as total ERK2 levels. Bottom, HEPG2 cells, 24 h after plating, were infected with empty vector control virus (CMV), a virus to express constitutively active AKT, a virus to express constitutively active MEK1 EE, or both the active AKT and active MEK1 EE viruses. Twenty-four hours after infection, cells were treated with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. Ninety-six hours after drug exposure, cells were isolated and viability was determined by trypan blue assay. Columns, percentage of trypan blue–positive cells; bars, SE. Data are from the mean of three independent studies. **C,** left, HEPG2 cells, 24 h after plating in triplicate, were transfected with NF-κB-luciferase (NF-κB-luc) and β-galactosidase (β-gal) constitutive reporter constructs. Parallel control plates of cells (data not shown) were transfected with NF-κB-luciferase and β-galactosidase constitutive reporter constructs and cotransfected with a plasmid to express dominant-negative IκB or with a plasmid to express a constitutively activated form of NF-κB as internal negative and positive controls. Thirty-six hours after transfection, cells were treated with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. At the indicated times, cells were isolated and the amount of luciferase per cell and the amount of β-galactosidase per cell were determined as described in Materials and Methods. Columns, from two separate studies; bars, SE. Control studies showed that overexpression of dominant-negative IκB blocked vorinostat-induced activation of NF-κB-luciferase activity (data not shown). Right, HEPG2 cells, 24 h after plating in triplicate, were infected with control empty vector virus (CMV) or a recombinant virus to express dominant-negative IκB S32A S36A (dnIκB). Twenty-four hours after infection, cells were treated with vehicle, sorafenib, vorinostat, or both sorafenib and vorinostat. Forty-eight hours after drug exposure, cells were isolated, spun onto glass slides, and stained using established methods for double-stranded DNA breaks indicative of apoptosis (TUNEL) as described in Materials and Methods. Columns, percentage of TUNEL-positive cells; bars, SE. Data are from a representative of two independent studies. #, P < 0.05, less cell killing than compared with the same condition in CMV-infected cells.
These findings argue that low doses of vorinostat and sorafenib induce activation of the extrinsic apoptotic pathway at the level of the CD95 death receptor and that the initial rapid loss of c-FLIP-s expression within 24 h occurs via activation of eIF2α and plays a critical role in transmitting death signals from the plasma membrane to the cytosol, resulting in a pleiotropic activation of multiple downstream apoptotic processes, including mitochondrial dysfunction.

Sorafenib was originally developed as an inhibitor of Raf family protein kinases but subsequently shown to be an inhibitor of several receptor tyrosine kinases and an inducer of ER stress signaling (12, 14, 21). Vorinostat has been shown to modulate in a concentration-dependent and cell type–dependent manner ERK1/2, NF-κB, AKT, JNK1/2, and p38 MAPK pathway signaling (23–25, 36–39). Furthermore, changes in NF-κB, ERK1/2, AKT, JNK1/2, and p38 MAPK signaling have been linked to the modulation of CD95 function and that of c-FLIP-s, expression, also in a cell type–dependent manner. These considerations prompted further examination of whether significant alterations in signaling pathway function occurred in vorinostat/sorafenib-treated cells and whether these changes could be related to CD95 activation, changes in apoptosis-regulatory protein expression, and overall tumor cell survival.

Twenty-four hours after drug exposure, a time at which CD95 activation had occurred, eIF2α phosphorylation had occurred, and the expression of multiple prosurvival proteins such as c-FLIP-s had already declined; no profound change in the basal activities of ERK1/2, AKT, JNK1/2, or p38 MAPK was
observed in HEPG2 or HEp3B cells (Fig. 5A; data not shown). Over the ensuing 72 h, activation status of the JNK1/2 or p38 MAPK pathways did not correlate strongly with cell death induction. In contrast to signaling pathways that promote death, activities of pathways that protect against cell death began to decline in cells treated with vorinostat and sorafenib 24 to 96 h after drug exposure. Within 48 h of exposure, ERK1/2 was inactivated in combined drug-exposed cells; within 72 h of exposure, AKT became inactivated. Inactivation of the ERK1/2 pathway was not due to the prior activation of CD95 or FADD, in view of the findings that knockdown of CD95 or FADD suppressed cell killing but did not maintain ERK1/2 phosphorylation (Supplementary Fig. S15). Thus, inactivation of ERK1/2 and AKT were relatively late events in cell death induction after sorafenib and vorinostat exposure but were not causally dependent on the primary CD95-dependent apoptotic signal.

Based on the findings in Fig. 5A, attempts were made to determine whether expression of constitutively active MEK1 and/or AKT protected cells from vorinostat and sorafenib exposure. Expression of constitutively active MEK1 maintained ERK1/2 phosphorylation in HEPG2 cells treated with vorinostat and sorafenib, as did expression of constitutively active AKT in maintaining levels of AKT S473 phosphorylation (Supplementary Fig. S16). Expression of either activated MEK1 or activated AKT almost abolished the toxicity of the individual drugs and significantly suppressed the toxicity of the drug combination (Fig. 5B, bottom). These findings correlated with maintenance of c-FLIP-s expression in tumor cells expressing activated MEK1 and activated AKT and treated with vorinostat and sorafenib (Fig. 5B, top). Collectively, these data further argue that maintained c-FLIP-s expression prevents CD95 signaling from activating the caspase-8-BID pathway to induce mitochondrial dysfunction and death. These findings also argue that primary activation of eIF2α followed by the secondary inhibition of ERK1/2 and AKT represents the likely sequence of events by which low doses of sorafenib and vorinostat suppress c-FLIP-s levels and subsequently maintain suppression of c-FLIP-s expression in transformed cells.

Prior studies using vorinostat have shown that this agent activates the transcription factor NF-κB and that this can act against the lethal actions of this drug (29, 36). Whether NF-κB function plays any role in cell survival after low-dose vorinostat and sorafenib treatment in our cell system was then examined. Treatment of HEPG2 and UOIK121LN cells with vorinostat caused a late post-24 h exposure-induced activation of NF-κB, which was not significantly altered by incubation of cells with low doses of sorafenib (Fig. 5C; Supplementary Fig. S17). As noted above, NF-κB activation following vorinostat treatment has been shown as a protective signal in malignant hematologic cells, we determined whether genetic inhibition of NF-κB function via expression of the super-repressor IκB S32A S36A altered the survival response of drug-treated carcinoma cells. Expression of IκB S32A S36A–inhibited vorinostat-induced activation of NF-κB and did not significantly alter the lethality of vorinostat as a single agent (Fig. 5C; Supplementary Fig. S17; data not shown). However, inhibition of NF-κB function significantly suppressed the death of cells treated with sorafenib and vorinostat. Our findings argue that hyperactivation of ERK1/2 and AKT can suppress killing that may be due to maintained expression of c-FLIP-s and that the observed late-phase activation of NF-κB induced by vorinostat treatment is, surprisingly, a toxic signal.

Finally, we did in vivo analyses using established ~150 mm³ HEp3B flank tumors to determine whether sorafenib and vorinostat interacted in a toxic manner in vivo. We noted that unselected clones of HEp3B and HEPG2 cells are poorly tumorigenic in the flanks of athymic mice and form tumors that rapidly become necrotic on growth beyond >200 mm³, potentially due to a relatively low CD31 staining (data not shown). As such, we chose an in vivo treatment, ex vivo colony formation assay approach, to assess tumor cell killing and long-term survival. A 3-day treatment of animals with either vorinostat or sorafenib caused little alteration in the cleavage status of caspase-3 or the TUNEL positivity of flank tumor sections (Fig. 5D, left). Combined exposure of animals/tumors to vorinostat and sorafenib caused a large increase in both the number of apoptotic TUNEL-positive cells and a large increase in immunoreactivity for the cleaved form of caspase-3. Combined, but not individual, exposure of animals/tumors to vorinostat and sorafenib caused a large increase in elf2α phosphorylation and large decreases in the expression of c-FLIP-s and MCL-1 and phosphorylation of AKT (S473). Of particular note, at the in vivo concentrations of sorafenib used as a single agent in our study, we observed near total inhibition of ERK1/2 phosphorylation by the drug, but this inhibition of ERK1/2 phosphorylation did not correlate with the cleavage of caspase-3, strong enhancement of TUNEL positivity, or profoundly lower c-FLIP-s or MCL-1 levels. In ex vivo colony formation assays using viable cells isolated from treated tumors after cessation of drug treatment, and with cells cultured in the absence of any drug in vitro, combined exposure of animals/tumors to sorafenib and vorinostat caused a greater reduction in cell survival of the explanted tumor cells growing ex vivo than was observed in the cells that had been exposed to either drug individually (Fig. 5D, right). Collectively, these findings argue that our molecularly defined markers for sorafenib and vorinostat lethality are observed in vitro and also in drug-treated tumors and that these effects correlate with an increase in both short-term and long-term tumor cell killing.

Discussion

Previous studies from our laboratories have shown that sorafenib and vorinostat interact in vitro in a greater than additive fashion to kill malignant hematologic cells. In chronic myelogenous leukemia cells, sorafenib- and vorinostat-induced cell killing correlated with decreased expression of the mitochondrial protective protein MCL-1 and the cyclin kinase inhibitor p21cip1/WAF1/mda6 (20). The present studies attempted to determine whether sorafenib and vorinostat also interacted to kill malignant epithelial cells attached to a substrate and, if so, to elucidate the mechanism responsible for this phenomenon.

The results of the present study indicate that low clinically relevant concentrations of sorafenib and vorinostat interact in a synergistic manner to kill liver, kidney, and pancreatic tumor cells in vitro. In vitro, the enhanced lethality of the regimen toward transformed cells was blocked by inhibition of CD95 or FADD function and abolished by overexpression of c-FLIP-s. Significantly, the protective effect of c-FLIP-s overexpression was observed despite the fact that sorafenib
and vorinostat, but not the drugs individually, suppressed expression of multiple downstream mitochondrial protective proteins, including BCL-2, BCL-XL, and MCL-1, and increased the expression/activity of multiple downstream mitochondrial toxic proteins, including BAX, BAK, BIM, BID, and BAD, events thought to act independently of CD95 and c-FLIP-s to also lower cell viability. These findings suggest that one central mechanism of sorafenib and vorinostat lethality in epithelial tumor cells involves primary activation of the extrinsic apoptosis pathway, in concert with diminished c-FLIP-s levels, which in turn facilitate secondary activation of a destabilized intrinsic apoptosis pathway (Supplementary Fig. S1B).

Inhibition of cathepsin B protease function also suppressed the toxicity of sorafenib and vorinostat. Cathepsin proteases are secreted enzymes as well as localized in the cell within acidic endosomes, and in some studies, cathepsins have been shown to promote cell death by cleaving the caspase-8 substrate BID independently of caspase-8, thereby causing mitochondrial dysfunction (e.g., ref. 31). In the present studies, overexpression of the caspase-8–specific inhibitor c-FLIP-s abolished drug-induced cell killing in liver, kidney, and pancreatic tumor cells, arguing that for cathepsin proteases to act as apoptotic mediators in sorafenib- and vorinostat-treated cells first requires activation of caspase-8. The expression and activity of cathepsin proteases is frequently increased in malignant cells and the secreted forms of these enzymes have been shown to play a key role in promoting invasion and angiogenesis, two critical characteristic features of the malignant phenotype (40, 41). Death receptor signaling (the extrinsic apoptosis pathway) is generally viewed as a pathway that proceeds via caspase-8 signaling to induce both BID cleavage and mitochondrial dysfunction or directly to procaspase-3 cleavage in both cases culminating in cell death (4–6). However, cathepsin proteases have also been shown to play a dynamic role in tumor necrosis factor-α and FAS-stimulated cell death processes wherein cathepsin proteases cooperate with caspase-8 to cause mitochondrial dysfunction and cell death (40, 41). Palacios et al. (42) recently showed that the cyclin-dependent kinase inhibitor flavopiridol (100 nmol/L) potentiated the lethality of tumor necrosis factor–related apoptosis-inducing ligand in MDA-MB-231 mammary tumor cells and that this effect was due to the suppression of c-FLIP-s/l expression by flavopiridol. Analogously, interactions between much higher concentrations of sorafenib and tumor necrosis factor–related apoptosis-inducing ligand in malignant hematopoietic cells have been related to inhibition of c-FLIP protein translation (43). Others, also using much higher concentrations of sorafenib, have observed c-FLIP and MCL-1 expression suppression by this agent, which is also likely due to profound suppression of transcription and translation (44). Inasmuch as cathepsins play a proapoptotic role in sorafenib- and vorinostat-treated cancer cells, such findings raise the possibility that tumor cells that survive exposure to these agents may do so by down-regulating cathepsin expression, which may result in diminished angiogenesis and a less invasive tumor. It also argues that inhibition of cyclin-dependent kinase 9 function or suppression of protein translation by sorafenib (compare our data showing increased elf2α phosphorylation; see also refs. 18, 19, 21), events that induce c-FLIP-s/l down-regulation, may represent a common mechanism by which vorinostat lethality is enhanced by sorafenib in carcinoma cells.

Prior studies from this group have shown that sorafenib in the ~10 to 15 μmol/L range, which are within pharmacologically achievable concentrations, induced cell death in human leukemic cells by promoting ER stress and diminishing expression of MCL-1 rather than by inhibiting either Raf family kinases or receptor tyrosine kinases (14–16, 20, 21). The present results in epithelial cancer cells showed that much lower concentrations of sorafenib (~3 μmol/L) as a single agent did not significantly alter MCL-1 levels or increase the phosphorylation of elf2α, as was also the case for vorinostat exposure. However, treatment of carcinoma cells with both sorafenib and vorinostat resulted in a large increase in the phosphorylation of PERK as well as its substrate elf2α. Collectively, these findings are suggestive of ER stress signaling. Findings in malignant hematologic cells argued strongly that down-regulation of the short-lived protein (half-life, 2-4 h) MCL-1 by translational inhibition played an important functional role in the lethality of the sorafenib and vorinostat drug combination. MCL-1 is a multidomain antiapoptotic member of the BCL-2 family that acts through several mechanisms, including cooperation with BCL-XL, to block mitochondrial outer membrane permeabilization by BAK and BAX and to prevent apoptosis (18). Translation inhibition of MCL-1 expression was independent of ERK1/2 inactivation. Notably, MCL-1 down-regulation was also observed in sorafenib/vorinostat-treated epithelial tumor cells in an elf2α-dependent fashion. Additional studies will be required to define the functional roles of ER stress signaling and the unfolded protein response in sorafenib and vorinostat lethality and relationship between these events, the activation of CD95 and enhanced CD95/FAS-L expression that occurred shortly following CD95 activation/DISC formation, and the rapid suppression of prosurvival protein expression (e.g., MCL-1 and c-FLIP-s) in malignant epithelial cells.

HDACIs, such as vorinostat, kill tumor cells through highly diverse mechanisms, including blocking histone deacetylation, and have been proposed to down-regulate/inactivate multiple oncogenic kinases by interfering with HSP90 function, leading to proteasomal degradation of these proteins. Prior studies in breast cancer cells have established that low (e.g., 500 nmol/L) concentrations of vorinostat, which are substantially below the 9 μmol/L Cmax of this agent, promoted activation of caspase-8 and that cleavage of BID was involved in drug lethality, in agreement with the present data involving liver, kidney, and pancreatic tumor cells (29). Disruption of signal transduction pathway function, reducing the activities within the Raf-MEK-ERK and phosphatidylinositol 3-kinase–phosphoinositide-dependent protein kinase 1–AKT pathways, will exert pleiotropic actions in tumor cells, including destabilization of c-FLIP-s and BCL family member proteins. Inhibition of these pathways was not readily measurable by immunoblotting over the initial 24 h after drug exposure but could, nevertheless, significantly contribute at later times to diminished expression of prosurvival molecules such as BCL-XL and MCL-1 and to increased expression of prodeath molecules such as BIM (3–6). Hence, although the initial insult of vorinostat and

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8 G. Zhang, M.A. Park, and P. Dent, unpublished observation.
sorafenib exposure caused CD95 activation and translational repression, shifting the apoptotic rheostat of a cell toward cell death and away from cell survival, the second destabilization of Raf-MEK-ERK and phosphatidylinositol 3-kinase-PDK1-AKT pathway function, whether by changes in HSP90 activity or other unknown factors, likely prevented any form of compensatory survival response being mounted by the cell resulting in the death response becoming “locked in place.”

Studies in leukemia and lymphoma cells have shown that vorinostat can enhance cell killing in these cells particularly when vorinostat-induced NF-κB activation has been suppressed via expression of a dominant-negative IκB protein (e.g., refs. 45–47). In leukemic cells, the actions of the extrinsic pathway in vorinostat lethality were not noted as a primary effector in the actions of vorinostat, although they could possibly play a role. We were surprised to discover, however, in hepatoma, renal, and pancreatic tumor cells that low-dose vorinostat-induced activation of NF-κB had little effect on cell survival, as previously observed in breast cancer cells, and that when combined with sorafenib, NF-κB activation facilitated the cell killing process (29). Activation of NF-κB in some cell systems has been linked to cell death processes, and NF-κB can increase the expression of death receptor ligands, such as tumor necrosis factor-α, at a transcriptional level (48, 49). Whether low doses of vorinostat increase tumor necrosis factor-α expression in epithelial tumor cells and whether this represents a portion of its toxic biology via activation of the extrinsic pathway independently of CD95 are beyond the scope of the present article.

In conclusion, our data indicate that sorafenib and vorinostat interact in a highly synergistic manner to kill liver, kidney, and pancreatic tumor cells in vitro. They also show that this effect translates into an in vivo model system using hepatoma cells growing in the flanks of athymic mice. Ongoing in vivo and animal studies and phase I studies in renal and liver cancer with these agents are presently defining in greater detail the mechanism(s) of action of these drugs and their putative clinical relevance.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

P. Dent thanks Dr. S. Lin for assisting Dr. G. Zhang in these studies and Dr. O. Korochinsky for assistance with NF-κB-luciferase assays and the training of Dr. M.A. Park for these assays. This manuscript is dedicated to the corresponding author’s Great Aunt Vera in her continued fight against renal cell carcinoma.

References


Vorinostat and Sorafenib Synergistically Kill Tumor Cells via FLIP Suppression and CD95 Activation

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