Sorafenib Activates CD95 and Promotes Autophagy and Cell Death via Src Family Kinases in Gastrointestinal Tumor Cells

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Abstract

Sorafenib and vorinostat interact in a synergistic fashion to kill carcinoma cells by activating CD95; the present studies have determined how sorafenib and vorinostat individually contribute to CD95 activation. Sorafenib (3–6 μmol/L) promoted a dose-dependent increase in Src Y416, ERBB1 Y845 and CD95 Y232/Y291 phosphorylation, and Src Y527 dephosphorylation. Low levels of sorafenib-induced (3 μmol/L) CD95 tyrosine phosphorylation did not promote surface localization whereas sorafenib (6 μmol/L), or sorafenib (3 μmol/L) and vorinostat (500 nmol/L) treatment promoted higher levels of CD95 phosphorylation which correlated with DISC formation, receptor surface localization, and autophagy. CD95 (Y232F, Y291F) was not tyrosine phosphorylated and was unable to localize plasma membrane or induce autophagy. Knockdown/knockout of Src family kinases abolished sorafenib-induced CD95 tyrosine phosphorylation, DISC formation, and the induction of cell death and autophagy. Knockdown of platelet-ived growth factor receptor-β enhanced Src Y416 and CD95 tyrosine phosphorylation, which correlated with elevated CD95 plasma membrane levels and autophagy, and with a reduced ability of sorafenib to promote CD95 membrane localization. Vorinostat increased reactive oxygen species levels, and in a delayed NFκB-dependent fashion, those of FAS ligand and CD95. Neutralization of FAS-L did not alter the initial rapid drug-induced activation of CD95; however, neutralization of FAS-L reduced sorafenib + vorinostat toxicity by ~50%. Thus, sorafenib contributes to CD95 activation by promoting receptor tyrosine phosphorylation, whereas vorinostat contributes to CD95 activation via the initial facilitation of reactive oxygen species generation and subsequently of FAS-L expression. Mol Cancer Ther; 9(8); 2220–31. ©2010 AACR.

Introduction

In the United States, hepatoma patients have a 5-year survival rate of less than 10% (1). We have recently developed a novel drug therapy combining the multikinase inhibitor sorafenib with the histone deacetylase inhibitor (HDACI) vorinostat, and this combination is entering phase I trial in hepatomas (2–5).

Sorafenib (Bay 43-9006, Nexavar; a RAF family kinase inhibitor) is a multikinase inhibitor that was originally developed as an inhibitor of RAF-1 but which was subsequently shown to inhibit multiple other kinases, including class III tyrosine kinase receptors (6). The antitumor effects of sorafenib in renal cell carcinoma and in hepatoma have been ascribed to the antiangiogenic actions of this agent through inhibition of the growth factor receptors (7–9). However, several groups, including ours, have shown in vitro that sorafenib kills human leukemia cells at concentrations below the maximum achievable dose (Cmax) of 15 to 20 μmol/L, through a mechanism involving the downregulation of MCL-1 (10, 11). Sorafenib-mediated MCL-1 downregulation occurred through a translational rather than a transcriptional or posttranslational process that was mediated by endoplasmic reticulum stress signaling (12, 13). This suggests that the previously observed antitumor effects of sorafenib are mediated by a combination of inhibition of RAF family kinases and the ERK1/2 pathway, receptor tyrosine kinases that signal angiogenesis, and the induction of endoplasmic reticulum stress signaling.

HDACI represent a class of agents that act by blocking histone deacetylation, thereby modifying chromatin structure and gene transcription. HDACs, along with histone acetyl-transferases, reciprocally regulate the acetylation status of the positively charged NH2-terminal histone tails of nucleosomes. HDACIs promote histone...
acetylation and neutralization of positively charged lysine residues on histone tails, allowing chromatin to assume a more open conformation, which favors transcription (14). However, HDACIs also induce the acetylation of other non-histone targets, actions that might have pleiotropic biological consequences, including inhibition of HSP90 function, induction of oxidative injury, and upregulation of death receptor expression (15–17). With respect to combinatorial drug studies with a multi-kinase inhibitor such as sorafenib, HDACIs are of interest in that they also downregulate multiple oncogenic kinases by interfering with HSP90 function, leading to the proteasomal 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_max of ~9 μmol/L (18–28).

We recently published that sorafenib and vorinostat interact to kill a wide range of tumor cell types via activation of the CD95 extrinsic apoptotic pathway, concomitant with drug-induced reduced expression of c-FLIP-s via PERK/erF2α activation (29, 30). The present studies have extended in greater molecular detail our analyses to understanding how sorafenib and vorinostat individually interact to promote CD95 activation and tumor cell death.

Materials and Methods

Materials
Sorafenib tosylate (Bayer) and vorinostat (Merck) were provided by the Cancer Treatment and Evaluation Program, National Cancer Institute/NIH (Bethesda, MD). Trypsin-EDTA, DMEM, RPMI, and penicillin-streptomycin were purchased from Life Technologies. HEPG2, HEP3B, and HuH7 (hepatoma) cells were purchased from American Type Culture Collection. Commercially available validated short hairpin RNA molecules to knock down RNA/protein levels were from Qiagen: CD95 (SI02654463; SI03118255), ATG5 (SI02655310), and Beclin 1 (SI00055573; SI00055587). For confirmatory purposes, we also used the short hairpin RNA construct targeting ATG5 (pLVTHM/Atg5), which was a generous gift from Dr. S. Yousefi, Department of Pharmacology, University of Bern, Bern, Switzerland. The reagents used and the performance of experimental procedures were described in refs. (2–5, 21, 29–37).

Methods

Culture and in vitro exposure of cells to drugs. All established cell lines were cultured at 37°C, 5% (v/v) CO2 in vitro using RPMI supplemented with 5% (v/v) FCS and 10% (v/v) nonessential amino acids. For short-term cell-killing assays and immunoblotting studies, cells were plated at a density of 3 × 10^3 per cm^2 (~2 × 10^3 cells per well of a 12-well plate), and 48 hours after plating, treated with various drugs, as indicated. In vitro vorinostat and sorafenib treatments were from 100 mmol/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 isolated at the indicated times, and 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, using a Becton Dickinson FACScan flow cytometer, the Annexin V/propidium iodide assay was carried out according to the instructions of the manufacturer to determine cell viability (BD Pharmingen). Vorinostat/sorafenib lethality, as determined by Annexin V/propidium iodide, was first evident ~24 hours after drug exposure (data not shown).

For SDS-PAGE and immunoblotting, cells were plated at 5 × 10^5 cells/cm^2 and treated with drugs at the indicated concentrations and after the indicated time of treatment, lysed in whole-cell lysis buffer [0.5 mol/L Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 1% β-mercaptoethanol, and 0.02% bromophenol blue], and the samples were boiled for 30 minutes. The boiled samples were loaded onto 10% to 14% SDS-PAGE and electrophoresis was run overnight. Proteins were electrophotorectified onto 0.22 μm of nitrocellulose, and immunoblotted with various primary antibodies against different proteins.

Transfection of cells with siRNA or with plasmids
For plasmids. Cells were plated as described above and 24 hours after plating, transfected. For mouse embryonic fibroblasts (2–5 μg), or other cell types (0.5 μg), plasmids expressing a specific mRNA (or siRNA) or appropriate vector control plasmid DNA was diluted in 50 μL of serum-free and antibiotic-free medium (one portion for each sample). Concurrently, 2 μL of 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 minutes. This mixture was added to each well/dish of cells containing 20 μL of serum-free and antibiotic-free medium for a total volume of 300 μL, and the cells were incubated for 4 hours at 37°C. An equal volume of 2× medium was then added to each well. Cells were incubated for 48 hours, then treated with vorinostat/sorafenib.

Transfection with siRNA. Cells were plated into 60 mm dishes from a fresh culture growing in log phase as described above, and transfected 24 hours after plating. Prior to transfection, the medium was aspirated and 1 mL of serum-free medium was added to each plate. For transfection, 10 nmol/L of the annealed siRNA, the positive sense control double-stranded siRNA targeting glyceraldehyde-3-phosphate dehydrogenase or the negative control (a “scrambled” sequence with no significant
homology to any known gene sequences from mouse, rat, or human cell lines were used. siRNA (10 nmol/L; scrambled or experimental) was added to this mixture and the solution was mixed by pipetting up and down several times. This solution was incubated at room temperature for 10 minutes, then added dropwise to each dish. The medium in each dish was swirled gently to mix, then incubated at 37°C for 2 hours. One milliliter of 10% (v/v) serum-containing medium was added to each plate, and cells were incubated at 37°C for 48 hours before replating (50 × 10^3 cells each) onto 12-well plates. Cells were allowed to attach overnight, then treated with vorinostat/sorafenib (0–48 hours). Trypan blue exclusion/TUNEL/flow cytometry assays and SDS-PAGE/immunoblotting analyses were done at the indicated time points.

**Microscopy for LC3-GFP expression.** Cells were transfected with a plasmid to express an LC3-GFP fusion protein, and were then cultured for 24 hours. Cells were then treated with drugs, as indicated. LC3-GFP–transfected cells were visualized at the indicated time points on a Zeiss Axiovert 200 microscope using an FITC filter.

**Recombinant adenoviral vectors; infection in vitro.** We generated and purchased previously noted recombinant adenoviruses to express a wide variety of proteins or to knock down p21 expression (Vector Biolabs). Cells were infected with these adenoviruses at an approximate multiplicity of infection of 50. Cells were incubated for 24 hours to ensure adequate expression of transduced gene products prior to drug exposures.

**Assessment of reactive oxygen species generation.** Hepatoma cells were plated in 96-well plates. Cells were preincubated with dihydro-DCF (5 mmol/L for 30 minutes) which is nonfluorescent in its dihydro form but, upon reaction with reactive oxygen species (ROS), becomes highly fluorescent. Dihydro-DCF is sensitive to oxidation by hydroxyl radicals and peroxy-nitrite directly and hydrogen peroxide in the presence of oxidases. Fluorescence measurements were obtained 0 to 30 minutes after the addition of the drug with a Vector 3 plate reader. Data are presented corrected for basal fluorescence of vehicle-treated cells at each time point and expressed as a fold increase in ROS levels. Each time point represents the mean of at least six data points per experiment and of a total of three independent experiments.

**Data analysis.** Comparison of the effects of various treatments was done using ANOVA and Student's t test. Differences with a P < 0.05 were considered statistically significant. Experiments shown are the means of multiple individual points (±SEM). Median dose-effect isobologram analyses to determine the synergism of drug interaction were done according to the methods of T-C. Chou and P. Talalay using the CalcuSyn program for Windows (Biosoft). Cells were treated with agents at a fixed concentration dose. A combination index value of <1.00 indicates synergy of interaction between the drugs, a value of 1.00 indicates additivity, and a value of >1.00 indicates antagonism of action between the agents.

**Results**

Treatment of gastrointestinal tumor cells (HEPG2, HEPA8, and UOK121LN) with low concentrations of sorafenib (3 and 6 μmol/L) increased the tyrosine phosphorylation of Src Y416, ERBB1 Y845, and the basal tyrosine phosphorylation of platelet-derived growth factor receptor-β (PDGFRβ) and caused Src Y527 dephosphorylation (Fig. 1A). Although sorafenib (3 μmol/L) modestly increased CD95 tyrosine phosphorylation, this level of phosphorylation neither promoted CD95 surface localization nor promoted caspase 8 association with CD95 (DISC formation; Fig. 1B, C, and D; Supplementary Fig. S1). Treatment of cells with a higher concentration of sorafenib (6 μmol/L) promoted a further increase in CD95 tyrosine phosphorylation above that induced by sorafenib (3 μmol/L), and which correlated with CD95 surface localization and promoted caspase 8 association with CD95 (Fig. 1B, C, and D). Concomitant treatment of cells with vorinostat enhanced sorafenib-induced (3 μmol/L) tyrosine phosphorylation of CD95 that correlated with CD95 surface localization and promoted caspase 8 association with CD95 (Fig. 1B, C, and D).

HuH7 hepatoma cells lack endogenous CD95 expression (29, 30, 37). Treatment of HuH7 cells with sorafenib (3 μmol/L) and vorinostat resulted in a very modest increase in tumor cell killing, which was significantly enhanced when HuH7 cells were transiently transfected with a plasmid to express wild-type CD95 (Fig. 2A). Transfection of cells with a plasmid to express a CD95 protein lacking the sites of tyrosine phosphorylation (Y232F, Y291F; Fig. 2B). In Fig. 1, we noted that sorafenib promoted the activation of Src and enhanced the basal levels of PDGFRβ tyrosine phosphorylation, and it has been shown in other cell systems that one of the protein kinases which phosphorylate CD95 is the Src family member c-Yes (37). Knockdown of PDGFRβ promoted a compensatory activation of Src within 24 hours as judged by the increased Y416 phosphorylation and the decreased Y527 phosphorylation (Fig. 2C). Knockdown of PDGFRβ enhanced CD95 tyrosine phosphorylation and procaspase 8 association with CD95, the phosphorylation and association of which, respectively, in the absence of PDGFRβ expression were not further enhanced by sorafenib (Fig. 2D, top). Enhanced levels of CD95 tyrosine phosphorylation caused by knockdown of PDGFRβ were blocked by the expression of a dominant negative (kinase inactive) Src protein (Fig. 2D, bottom).

To confirm our findings in hepatoma cells using other genetic approaches, we used the SV40 large T-antigen
transformed mouse embryonic fibroblasts lacking expression of c-Src, c-Fyn, and c-Yes. In transformed MEFs; sorafenib (3 μmol/L) and vorinostat treatment, or sorafenib (6 μmol/L) treatment, promoted surface localization of CD95 in a Src family kinase-dependent fashion (Fig. 3A). Transfection of wild-type MEFs with a plasmid to express wild-type CD95, followed by sorafenib (6 μmol/L) exposure resulted in enhanced CD95 tyrosine phosphorylation and enhanced DISC formation (Fig. 3B). In MEFs lacking expression of c-Src, c-Fyn, and c-Yes, sorafenib (6 μmol/L) exposure did not cause CD95 tyrosine phosphorylation or DISC formation. The toxicity of sorafenib was reduced in fibroblasts lacking expression of c-Src, c-Fyn, and c-Yes (Fig. 3C). In hepatoma cells, expression of dominant negative Src suppressed sorafenib-induced (6 μmol/L) CD95 activation; dominant negative Src increased basal levels of cell death but suppressed drug-induced toxicity (Fig. 3D).

Prior studies by our group had shown that sorafenib and vorinostat-induced activation of CD95 increased the levels of a protective form of autophagy (29, 30, 36). In agreement with data in Fig. 2D showing that knockdown of PDGFRβ increased CD95 tyrosine phosphorylation; knockdown of PDGFRβ increased basal levels of plasma membrane–associated CD95 and suppressed the ability of sorafenib (6 μmol/L) to promote CD95 plasma membrane levels (Fig. 4A). Knockdown of PDGFRβ or treatment with sorafenib (6 μmol/L) increased autophagy in a CD95- and ATG5-dependent fashion (Fig. 4B). The ability of sorafenib to kill hepatoma cells was reduced by the knockdown of PDGFRβ and sorafenib toxicity (6 μmol/L) was suppressed by the knockdown of CD95 (Fig. 4C). Expression of wild-type CD95, but not mutant CD95 (Y232F, Y291F), facilitated sorafenib-induced (6 μmol/L) autophagy in HuH7 cells (Supplementary Fig. S2). In agreement with the above findings, the expression of dominant negative Src suppressed sorafenib-induced (6 μmol/L) autophagy as well as tumor cell killing (Figs. 3D and 4D). In ATG5−/− MEFs, which cannot undergo autophagy, sorafenib- and vorinostat-induced...
toxicities were enhanced (Supplementary Fig. S3). Vorinostat treatment of malignant blood cancer cells strongly and rapidly increases p21Cip-1/WAF1/mda-6 (p21) expression; however, in hepatoma cells, it more modestly elevated p21 levels and did so in a delayed fashion (Supplementary Fig. S4, top blot; refs. 20, 21). Increased p21 levels were reduced by cotreatment with sorafenib. Knockdown of p21 reduced sorafenib + vorinostat toxicity and reduced drug-induced autophagy (Supplementary Fig. S4, bottom graphs). These findings are similar to our prior studies in primary hepatocytes treated with bile acids, in which over-expression of p21 promoted increased CD95 expression and activation, and loss of p21 reduced CD95 activation and bile acid–induced autophagy (36). Knockdown of p21 suppressed the ability of sorafenib + vorinostat treatment to increase CD95 surface localization and DISC formation (Supplementary Fig. S4, immunohistochemistry).

We next determined how vorinostat promoted sorafenib-induced CD95 activation. Sorafenib (3 μmol/L) and vorinostat (500 nmol/L) rapidly promoted the generation of ROS in HEPG2 cells that was quenched by the expression of thioredoxin (Supplementary Fig. S5). Similar data were obtained in HEP3B and UOK121LN cells (data not shown). Quenching drug-induced ROS suppressed sorafenib and vorinostat toxicity and blocked CD95 activation (Fig. 5A); sorafenib and vorinostat increase cell

Figure 2. Sorafenib-induced Src-dependent CD95 tyrosine phosphorylation is due to inhibition of PDGFRβ. A, HuH7 cells were transfected with empty vector plasmid (CMV), a plasmid to express CD95-YFP, or a plasmid to express mutant inactive CD95-YFP (Y232F, Y291F). Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (3 μmol/L) and vorinostat (500 nmol/L). Cells were isolated 48 h after exposure and viability determined by trypan blue exclusion (n = 3, ±SEM). B, HuH7 cells were transfected with a plasmid to express CD95-YFP or a plasmid to express mutant inactive CD95-YFP (Y232F, Y291F) and 24 h later were treated with vehicle (DMSO) or sorafenib (3 μmol/L) and vorinostat (500 nmol/L). Cells were isolated 6 h after exposure and CD95 immunoprecipitated. The amount of caspase 8 association with CD95 was determined. Representative blots (n = 3). C, HEPG2 cells were transfected with scrambled siRNA (siSCR) or an siRNA to knock down PDGFRβ. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (6 μmol/L). Cells were isolated 30 min later and the phosphorylation of Src determined by immunoblotting. Representative blots (n = 3). D, top blots: HEPG2 cells were transfected with scrambled siRNA (siSCR) or an siRNA to knock down PDGFRβ. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (6 μmol/L). Cells were isolated 6 h later and CD95 immunoprecipitated. The association of caspase 8 with CD95 and the tyrosine phosphorylation of CD95 were determined. Representative blots (n = 3). Bottom blots: cells were transfected with scrambled siRNA (siSCR) or an siRNA to knock down PDGFRβ and in parallel were transfected with an empty vector plasmid (CMV) or a plasmid to express dominant negative c-Src. Thirty-six hours after transfection, cells were isolated and CD95 immunoprecipitated. The tyrosine phosphorylation of CD95 was determined. Representative blots (n = 3).
Figure 3. Src family kinase signaling is essential for sorafenib-induced CD95 activation and drug toxicity. A, wild-type or Src/Fyn/Yes null SV40 large T transformed MEFs plated in eight-well chamber slides and were treated with vehicle (DMSO), sorafenib (3 and 6 μmol/L), or vorinostat (500 nmol/L), as indicated. Cells were fixed 6 h after exposure and surface levels of CD95 determined by immunohistochemistry. The density of CD95 staining was determined in 40 cells (n = 2, ±SEM). B, wild-type or Src/Fyn/Yes null SV40 large T transformed MEFs were transfected with a plasmid to express CD95-YFP or a plasmid to express mutant inactive CD95-YFP (Y232F, Y291F) and 24 h later were treated with vehicle (DMSO) or sorafenib (6 μmol/L). Cells were isolated 6 h later and CD95 immunoprecipitated via the YFP tag. The association of caspase 8 with CD95 and the tyrosine phosphorylation of CD95 were determined. Representative blots (n = 3). C, wild-type or Src/Fyn/Yes null SV40 large T transformed MEFs were treated with vehicle (DMSO) or increasing concentrations of sorafenib (0.1–6.0 μmol/L). Cells were isolated 48 h after drug exposure and viability determined by trypan blue exclusion (n = 3, ±SEM). D, HEPG2 cells were transfected with empty vector plasmid (CMV) or a plasmid to express dominant negative Src. Twenty-four hours after transfection, cells were treated with sorafenib (3 and 6 μmol/L), as indicated. Top, cells were isolated 6 h after drug exposure, CD95 immunoprecipitated and the levels of CD95 tyrosine phosphorylation and the association of procaspase 8 with CD95 determined. Representative blots (n = 3). Bottom, cells were isolated 48 h after treatment and viability determined by trypan blue exclusion (n = 3, ±SEM).
Figure 4. PDGFRβ regulates CD95-dependent sorafenib-induced autophagy. A, HEPG2 cells were plated in eight-well chamber slides and transfected with scrambled siRNA (siSCR) or an siRNA to knock down PDGFRβ. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (6 μmol/L). Cells were fixed 6 h after exposure and surface levels of CD95 determined by immunohistochemistry. The density of CD95 staining was determined in 40 cells (n = 2, ±SEM). B, HEPG2 cells were plated in eight-well chamber slides and transfected with a plasmid to express LC3-GFP and in parallel with scrambled siRNA (siSCR) or siRNA molecules to knock down PDGFRβ or CD95. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (6 μmol/L). The number of intense punctate GFP-LC3 staining vesicles was determined in 40 cells (n = 2, ±SEM). C, HEPG2 cells were transfected with scrambled siRNA (siSCR) or siRNA molecules to knock down PDGFRβ or CD95. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (6 μmol/L). Forty-eight hours later, cells were isolated and viability determined by trypan blue exclusion (n = 3, ±SEM). D, HEPG2 cells were plated in eight-well chamber slides and transfected with a plasmid to express LC3-GFP and in parallel with a plasmid to express dominant negative Src. Thirty-six hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (6 μmol/L). The number of intense punctate GFP-LC3 staining vesicles was determined in 40 cells (n = 2, ±SEM).
surface CD95 levels by 1.97 ± 0.08-fold, which was reduced by transfection of thioredoxin to a 1.12 ± 0.06-fold increase (P < 0.05). In agreement with our CD95 activation data, and the role CD95 plays in stimulating autophagy, quenching of ROS also blocked drug combination–induced autophagy (Supplementary Fig. S6).

Treatment of hepatoma cells with sorafenib and vorinostat initially suppressed total cellular protein tyrosine phosphatase activity by ~25%, which was blocked by the expression of thioredoxin (Fig. 5B). However, within 3 hours, vorinostat and sorafenib treatment profoundly enhanced tyrosine phosphatase activity, again, in an ROS-dependent fashion. The ability of vorinostat and sorafenib to induce CD95 tyrosine phosphorylation in multiple gastrointestinal tumor types was blocked by the expression of thioredoxin (Fig. 5C).

In prior studies, we noted that ~6 to ~24 hours following vorinostat exposure, hepatoma cells increased the expression of FAS ligand and CD95 in a cell type–dependent fashion (29). Incubation of hepatoma cells with an anti-FAS-L neutralizing antibody did not alter the ability of sorafenib (3 μmol/L) and vorinostat treatment to activate CD95 6 hours after drug exposure (Fig. 6A, immunohistochemistry, top). However, neutralization of FAS-L suppressed the ability of sorafenib (3 μmol/L) and vorinostat treatment to kill tumor cells by ~50% (Fig. 6A, bottom graph). Vorinostat activates NFκB, an effect that is modestly enhanced by sorafenib (3 μmol/L), and an effect that was blocked by the expression of dominant negative IκB super-repressor (Fig. 6B, left graph; data not shown). Expression of the dominant negative IκB super-repressor suppressed drug-induced tumor cell killing (Fig. 6B, right graph). Vorinostat activates NFκB, an effect that is modestly enhanced by sorafenib (3 μmol/L), and an effect that was blocked by the expression of dominant negative IκB super-repressor (Fig. 6B, left graph; data not shown). Expression of the dominant negative IκB super-repressor suppressed drug-induced tumor cell killing (Fig. 6B, right graph).

Figure 5. Vorinostat-induced ROS plays a key role in promoting PTPase inactivation and CD95 tyrosine phosphorylation. A, HEPG2 cells were transfected with empty vector plasmid (CMV) or a plasmid to express thioredoxin (TRX). Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (3 μmol/L) and vorinostat (500 nmol/L). Cells were isolated 48 h after exposure and viability determined by trypan blue exclusion (n = 2, ±SEM). B, HEPG2 cells were plated in 96-well plates and 24 h after plating were transfected with empty vector plasmid (CMV) or a plasmid to express thioredoxin. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or sorafenib (3 μmol/L) and vorinostat (500 nmol/L). PTPase activity was measured using a commercial kit as described in Methods (n = 2, ±SEM). C, left, HuH7 cells transfected with plasmids to express CD95-YFP or CD95-YFP FF. Twenty-four hours after plating in eight-well chamber, slides were treated with vehicle (DMSO) or sorafenib (3 μmol/L) and vorinostat (500 nmol/L) in combination. Cells were fixed after 6 h and cell surface CD95 levels determined. Right, HEPG2 cells were transfected with empty vector (CMV) or to express wild-type thioredoxin. Twenty-four hours after transfection, cells were treated with vehicle (DMSO) or with sorafenib (3.0 μmol/L) and vorinostat (500 nmol/L). Cells were isolated after 6 h and CD95 immunoprecipitated to determine DISC formation and CD95 tyrosine phosphorylation (n = 3, ±SEM).
Figure 6. Vorinostat promotes sorafenib toxicity by increasing expression of FAS-L. A, top immunohistochemistry, HEPG2 cells plated in eight-well chamber slides were pretreated with control IgG or an IgG to neutralize FAS-L (1 μg/mL) and 30 min later treated with vehicle (DMSO) or sorafenib (3 μmol/L) and vorinostat (500 nmol/L) in combination. Cells were fixed 6 h after exposure and surface levels of CD95 determined by immunohistochemistry. The density of CD95 staining was determined in 40 cells (n = 2, ±SEM). Bottom graph, HEPG2 cells were pretreated with control IgG or an IgG to neutralize FAS-L (1 μg/mL) and 30 min later treated with vehicle (DMSO) or vorinostat (500 nmol/L) in combination. Forty-eight hours later, cells were isolated and viability determined by trypan blue exclusion (n = 3, ±SEM). B, left graph, HEPG2 cells 24 h after plating in 96-well plates were transfected with NFκB-luciferase and β-galactosidase constitutive reporter constructs. Thirty-six hours after transfection, cells were treated with vehicle (DMSO), sorafenib (3.0 μmol/L), vorinostat (500 nmol/L), or both sorafenib and vorinostat. Cells were assayed for NFκB-luciferase and β-galactosidase activity 24 h after treatment (±SEM, a representative from two separate studies). Control studies showed that overexpression of dominant negative IκB blocked vorinostat-induced activation of NFκB-luciferase activity (data not shown). Right graph, HEPG2 cells 24 h after plating were infected with control empty vector virus (CMV) or a recombinant virus to express dominant negative IκB S32A S36A (dn IκB). Twenty-four hours after infection, cells were treated with vehicle (DMSO), sorafenib (3.0 μmol/L), vorinostat (500 nmol/L), 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 (n = 2, ±SEM). C, HEPG2 and HEPA3B cells 24 h after plating were infected with control empty vector virus (CMV) or a recombinant virus to express dominant negative IκB S32A S36A (dn IκB). Twenty-four hours after infection, cells were treated with vehicle (DMSO) or vorinostat (500 nmol/L). Cells were isolated 12 and 24 h after treatment. SDS-PAGE and immunoblotting were done to determine changes in the expression of CD95 and FAS-L, as indicated. A representative is shown of three separate studies.
In other systems, it has been shown that HDACIs could, via NF-κB, increase the expression of death receptors and their cognate ligands (38). We have previously published that vorinostat induced expression of FAS-L and CD95 in hepatoma cells and we now note that this effect was blocked by the expression of dominant negative IκBα (Fig. 6C; ref. 29). Collectively, our data argue that vorinostat contributes to sorafenib-induced activation of CD95 shortly following drug exposure by elevating ROS levels that inhibit PTPase function and promote CD95 activation, whereas at later time points, vorinostat promotes CD95 signaling and tumor cell death by increasing FAS-L expression in an NF-κB-dependent fashion.

**Discussion**

Sorafenib is a small molecule drug that inhibits RAF family serine/threonine kinases and class III receptor tyrosine kinases such as the PDGF and FLT receptors. Previous studies have shown that sorafenib and the HDACI vorinostat interact *in vitro* and *in vivo* in a greater than additive fashion to kill transformed cells that was mechanistically dependent on activation of CD95 (29, 30). The present studies attempted to determine, in much greater detail, the molecular mechanisms by which sorafenib and vorinostat, as individual agents, interacted to activate CD95 and promote drug toxicity.

Sorafenib (3–6 μmol/L) caused a dose-dependent increase in CD95 tyrosine phosphorylation and, based on multiple criteria, in parallel activated Src family nonreceptor tyrosine kinases. Expression of dominant negative Src or knockout of Src/Fyn/Yes blocked sorafenib-induced CD95 tyrosine phosphorylation. CD95 (Y232F, Y291F) was not activated by either higher sorafenib (6 μmol/L) concentrations or by sorafenib (3 μmol/L) and vorinostat treatment. Knockdown of class III receptor tyrosine kinase PDGFRβ expression enhanced Src activity; in a Src-dependent fashion, knockdown of PDGFRβ enhanced CD95 tyrosine phosphorylation and also suppressed the ability of sorafenib to induce CD95 tyrosine phosphorylation. Vorinostat rapidly enhanced the ability of lower sorafenib concentrations to increase ROS levels and to transiently suppress total cellular PTPase activity within 1 hour, which was due to ROS generation. However, ROS generation also significantly enhanced PTPase activity 3 hours after drug treatment and correlated with Src Y527 dephosphorylation and with vorinostat-stimulated CD95 tyrosine phosphorylation. Thus, one potential model for molecular drug action is that sorafenib-mediated inhibition of PDGFRβ causes a compensatory activation of Src family tyrosine kinases that in turn phosphorylate and activate CD95. In addition, based on the degree to which PDGFRβ is inhibited by sorafenib, CD95 becomes tyrosine-phosphorylated in a Src-dependent fashion. Vorinostat, by interacting with sorafenib to elevate ROS levels, rapidly suppresses cellular PTPase activity which promotes greater levels of CD95 tyrosine phosphorylation and CD95 activation.

Knockdown of PDGFRβ increased basal plasma membrane levels of CD95 that was Src-dependent, and when PDGFRβ was knocked down, the relative ability of sorafenib to cause further activation of CD95 was reduced. This finding was mirrored by changes in autophagy presented in Fig. 4B, wherein knockdown of PDGFRβ increased autophagy in a CD95-dependent fashion and in cells lacking PDGFRβ, the ability of sorafenib to cause additional autophagy was reduced. Although we observed enhanced levels of CD95 activation after PDGFRβ knockdown in Fig. 4A, this did not translate into increased basal levels of cell killing in Fig. 4C; possibly this was because we were observing elevated levels of protective autophagy after knockdown as was shown in Fig. 4B (29, 30). Thus, logically, based on data in Fig. 4A and B, the relative reduction in sorafenib-induced cell killing in PDGFRβ knockdown cells in Fig. 4C was because without expression of the key target, i.e., PDGFRβ, sorafenib cannot instantaneously initiate the series of events that would lead to PDGFRβ inhibition, compensatory Src activation, CD95 tyrosine phosphorylation, etc.

Based on our data, if sorafenib is promoting the activation of Src family kinases, we reasoned that it is probable that other targets of Src kinases are also phosphorylated in response to sorafenib treatment. For example, in a preliminary study, we noted that FAK and IGF1R tyrosine phosphorylation are enhanced in response to sorafenib exposure. Increased FAK signaling has the potential to promote metastatic spread of tumor cells via Src (39). Signaling by Src kinases is known to facilitate ERBB1 Y845 phosphorylation and we observed this in our system following sorafenib treatment. Phosphorylation of Y845 has been linked to ERBB1-induced tumor cell growth, independent of ERK1/2 and phosphoinositide-3-kinase signaling (40). In at least one study, low concentrations of sorafenib have been shown to promote MEK1/2 activation via IGF1 receptor signaling, and inhibition of MEK1/2 signaling enhanced sorafenib toxicity (41). We have recently published similar data in malignant blood cancer cells (42). Thus, the practical outcome of sorafenib promoting Src kinase activation is that while this drug acts to suppress tumor growth through Src-dependent activation of CD95, the induction of endoplasmic reticulum stress and inhibition of proangiogenic growth factor receptors; it also has the capacity to promote tumor cell survival and migration through elevated signaling by Src, FAK, ERBB1, IGF1R, and possibly, the ERK1/2 pathway. Additional studies will be required to define the precise nodal enzymes within the sorafenib-induced signaling network that can promote and suppress sorafenib lethality.

We recently noted, using higher concentrations of vorinostat than those used in this article, that the drug induced DNA damage, via ROS generation, and this played a key role in the activation of NF-κB by the drug (43). Vorinostat facilitated CD95 activation in hepatoma cells by two mechanisms: increased CD95 tyrosine phosphorylation and increased expression of FAS-L.
Vorinostat induced ROS generation, and when in combination with sorafenib, inhibited PTPase activity and then profoundly activated PTPase activity; effects that were both ROS-dependent. These effects also correlated with increased CD95 phosphorylation, increased Src Y416 phosphorylation, and dephosphorylation of Src Y527. PTPases have a ~10-fold higher specific activity than the protein kinases, the actions of which are reversed, meaning that a small reduction in PTPase activity could significantly modify a site of phosphorylation which is being actively increased due to the actions of a kinase (44). The precise PTPase that regulates CD95 tyrosine phosphorylation or the Src Y527 phosphorylation in hepatoma cells has not been identified, although it has been shown that both CD45 and SHP1 could proximally modulate CD95 signaling in immune cell types, and that both SHP1 and Src family kinases could associate with CD95 (45, 46). This data, combined with our present findings, argues that specific inhibitors of SHP-1 could be of value in promoting sorafenib toxicity.

Using a neutralizing antibody, inhibition of FAS-L function suppressed sorafenib + vorinostat toxicity by ~50%. This correlated with vorinostat, in an NFκB-dependent fashion, promoting increased expression of FAS-L 12 to 24 hours after drug exposure. However, a multitude of experiments have shown that NFκB activation could also stimulate the expression of antiapoptotic proteins and promote cell growth. Previously, we have shown that the rapidly (~6 hours) induced activation of CD95 was due, in part, to ceramide generation, and our present findings showed that CD95 activation at this time point was insensitive to the neutralizing antibody. Multiple HDACIs have shown an increase in the protein levels of FAS-L and/or CD95 (e.g., refs. 38, 47), and this has been suggested as one important mechanism for the antitumor effects of HDACIs.

One well-described additional action of HDACIs in tumor cells is to increase expression of the cyclin dependent kinase inhibitor p21Cip-1/WAF1/mda-6 (p21; refs. 20, 29, 43). We noted that vorinostat, in contrast with our findings in malignant blood cancer cells, modestly increased p21 levels but could also act to promote tumor cell survival through NFκB activation. Thus, sorafenib, in part, subverts vorinostat-induced cell killing processes by blocking increased p21 expression. Of note was our finding that sorafenib suppressed vorinostat-induced p21 expression but not the enhancement of FAS-L expression; because p21 protein levels, unlike those of FAS-L, are regulated by both protein and mRNA stabilization as well as for both proteins at the level of transcription, our data suggests that the HDAC1-induced expression of p21 most probably occurs at a posttranscriptional level (48, 49).

We have previously shown that sorafenib and vorinostat combination therapy are effective at killing hepatoma cells in vivo, and sorafenib and HDAC1 combination therapy is entering phase I evaluation in patients with hepatoma, as well as in patients with renal carcinoma and non–small cell lung cancer (29, 30). The present studies provide additional mechanistic information as to how these agents interact and, furthermore, predict that inhibitors of survival signaling receptors, e.g., ERBB1 and IGFR, which are activated in response to vorinostat exposure, will enhance the antitumor efficacy of this drug combination.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

PHS grants R01-DK32825, R01-CA104177, and R01-CA108520 (P. Dent); PHS grants R01-CA67533, R01-CA77141, and R01-CA93738 (S. Grant); and a Leukemia Society of America grant 6405-97. These studies were also funded in part by The Jimmy V Foundation.

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Received 03/19/2010; revised 05/24/2010; accepted 06/11/2010; published OnlineFirst 08/03/2010.

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2230 Mol Cancer Ther, 9(8) August 2010

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Published OnlineFirst August 3, 2010; DOI: 10.1158/1535-7163.MCT-10-0274

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Molecular Cancer Therapeutics

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doi:10.1158/1535-7163.MCT-10-0274

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