Sorafenib Suppresses JNK-Dependent Apoptosis through Inhibition of ZAK

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Introduction

Sorafenib is a U.S. Food and Drug Administration–approved for the treatment of renal cell carcinoma and hepatocellular carcinoma and has been combined with numerous other targeted therapies and chemotherapies in the treatment of many cancers. Unfortunately, as with other RAF inhibitors, patients treated with sorafenib have a 5% to 10% rate of developing cutaneous squamous cell carcinoma (cSCC)/keratoacanthomas. Paradoxical activation of extracellular signal–regulated kinase (ERK) in BRAF wild-type cells has been implicated in RAF inhibitor–induced cSCC. Here, we report that sorafenib suppresses UV-induced apoptosis specifically by inhibiting c-jun–NH2–kinase (JNK) activation through the off-target inhibition of leucine zipper and sterile alpha motif–containing kinase (ZAK). Our results implicate suppression of JNK signaling, independent of the ERK pathway, as an additional mechanism of adverse effects of sorafenib. This has broad implications for combination therapies using sorafenib with other modalities that induce apoptosis.

Abstract

Sorafenib is U.S. Food and Drug Administration–approved for the treatment of renal cell carcinoma and hepatocellular carcinoma and has been combined with numerous other targeted therapies and chemotherapies in the treatment of many cancers. Unfortunately, as with other RAF inhibitors, patients treated with sorafenib have a 5% to 10% rate of developing cutaneous squamous cell carcinoma (cSCC)/keratoacanthomas. Paradoxical activation of extracellular signal–regulated kinase (ERK) in BRAF wild-type cells has been implicated in RAF inhibitor–induced cSCC. Here, we report that sorafenib suppresses UV-induced apoptosis specifically by inhibiting c-jun–NH2–kinase (JNK) activation through the off-target inhibition of leucine zipper and sterile alpha motif–containing kinase (ZAK). Our results implicate suppression of JNK signaling, independent of the ERK pathway, as an additional mechanism of adverse effects of sorafenib. This has broad implications for combination therapies using sorafenib with other modalities that induce apoptosis.

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Type Culture Collection (ATCC) fingerprints (ATCC.org), to the Cell Line Integrated Molecular Authentication database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/; Nucleic Acids Research 37:D925-D932 PMC1: PMC2686526) and to the MD Anderson Cancer Center fingerprint database. Normal human epidermal keratinocytes (NHEK) were obtained from Lonza in June 2012, aliquoted, and used according to the manufacturer’s instructions, and not further tested.

Cell culture and UV irradiation
HaCaT cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F-12 50/50 (Cellgro) supplemented with 10% FBS (Sigma), glutamine, and Primocin (Invivogen). NHEKs (Lonza) were cultured in media according to the manufacturer’s instructions. Irradiation was performed using an FS40 sunlamp doped by an IL1700 radiometer. Following irradiation, cells were treated with sorafenib (LC Laboratories) or dimethyl sulfoxide (DMSO; 1:2,000).

Flow cytometry
Adherent cells were trypsinized and nonadherent cells were collected for staining with Annexin V, TMRE (tetra-methylrhodamine), and Cytotox Blue. TMRE (Invitrogen) was used as a measure of mitochondrial membrane potential, Annexin V–APC (Invitrogen) as a probe for apoptosis, and Cytotox Blue (Invitrogen) as an indicator for dead cells. Data were collected and analyzed using a flow cytometer (FACScalibur; Becton Dickinson) and FlowJo Software (Tree Star). Data were calculated and charts were plotted using GraphPad Prism 5 software.

Western blot analysis
Cell were lysed in standard buffers with protease inhibitors (Roche) and phosphatase inhibitors (Santa Cruz Biotechnology) with extracts run on SDS/PAGEs and transferred to Immobilon-P transfer membrane (Millipore). Western blot analysis was blocked in TBST (10 mmol/L Tris-HC1 pH8, 150 mmol/L NaCl, 0.5% Tween) containing 2.5% bovine serum albumin, probed with primary antibodies (all Cell Signaling Technology), corresponding horseradish peroxidase–conjugated secondary antibodies, and signals detected using the ECL Kit (Amersham).

Lentiviral knockdown experiments
Lentiviral short hairpin RNA (shRNA) knockdown was accomplished using standard lentiviral methods using 293T cells and psPAX2 and VSVG packaging plasmids. shRNA clones against leucine zipper and sterile alpha motif–containing kinase (ZAK; clones: V2LHS_239842; V3LHS_336769) and TAOK2 (clone: V3LHS_315551), as well as a nonsilencing shRNA were obtained from Open Biosystems in the GIPZ vector. Following transduction, cells were puromycin-selected to obtain a stably infected population and fluorescence-activated cell sorted (FACS) to obtain cells with high-level suppression. Degree of mRNA suppression was quantified by quantitative PCR using TaqMan probes using internally controlled (two-color, same well) GAPDH (glyceraldehyde-3-phosphate dehydrogenase) probes to ensure proper normalization.

Mice experiments
For chronically irradiated SKH-1E hairless mice (Charles River Laboratories), 3-month-old female mice were irradiated thrice weekly for a total weekly dose of 12.5 kJ/m² UVB (solar simulator; Oriel). At 72 days, sorafenib treatment was started by oral gavage (12.5% Cremphor, 12.5% ethanol in water) at 50 mg/kg/d.

Immunohistochemistry
cSCCs biopsied from patients treated with or without sorafenib were obtained under Institutional Review Board approval (LAB08-0750). Staining levels were quantified by counting positively labeled cells and dividing by the total area of the tumor tissue (malignant keratinocytes) within each sample. To measure tumor areas, all samples were photographed, tumor cells outlined, and total pixel numbers calculated using image analysis tools in Adobe Photoshop and standardized to a hemacytometer to convert to mm².

Soft agar assays
For growth assays, transformed and immortal cells were generated by retroviral infection. Wild-type (WT) mouse embryonic fibroblasts (MEF) and Craf-deficient (Craf–/–) MEFs infected with HRAS (pBABE puro HRASV12) and E1A (pLS adenovirus E1A-125) expressing lentiviruses were selected with puromycin. Cells were seeded in soft agar at 2 × 10³ in 24-well plate format in growth medium containing 0.3% agar over a base layer of 0.6% agar, with varying concentrations of sorafenib or with DMSO (Sigma–Aldrich) vehicle control.

Results
Sorafenib suppresses UV-induced apoptosis in keratinocytes
Because many of the cSCC/keratoacanthoma induced by RAFi occur on sun-exposed areas, we hypothesized instead that sorafenib could affect UV-induced apoptosis. When we exposed HaCaT keratinocyte cell lines and primary NHEKs to 300 J/m² of UVB, they underwent significant apoptosis by 24 hours (Fig. 1A and B) as this suppression of apoptosis could also be observed by assessing cleaved caspase-3 levels by Western blot analysis (Supplementary Fig. S1). The stress-activated c-jun–NH2–kinase N-terminal (JNK) mitogen-activated protein kinase (MAPK) pathway is critically important in UV-induced apoptosis (20), so we...
explored its activation in cells treated with and without sorafenib after UV irradiation. Phospho-JNK levels were highly induced in DMSO-treated cells following UV irradiation, and potently suppressed in the presence of 1 μmol/L sorafenib in both HaCaT (Fig. 1C) and NHEK (Fig. 1D) cells. Phosphorylation of MKK4 (MAP2K4) and MKK7 (MAP2K7), two kinases required for the activation of JNK (21–23), were also upregulated significantly by UV treatment particularly at 1 and 6 hours (lanes 3, 5, and 7; Fig. 1C and D) and suppressed by drug treatment (lanes 4, 6, and 8; Fig. 1C). ERK signaling, as seen by phospho-ERK levels, remained intact, with paradoxical hyperactivation in the presence of sorafenib was observed, particularly in HaCaT cells. Importantly, phospho-MKK4 and phospho-MKK7, upstream kinases that activate JNK, were both strongly induced by UV radiation and strongly suppressed by sorafenib.

Sorafenib suppresses apoptosis and JNK signaling independently of ERK activation

Because sorafenib exerts potent effects on ERK signaling (5, 14), we sought to probe whether its suppression of JNK-dependent apoptosis could be the result of cross talk between ERK and JNK signaling. Because sorafenib induces a dose-dependent activation of the MAPK pathway in BRAF wild-type cells (5, 14), we separated the effects on ERK signaling by simultaneously treating cells with the MEK inhibitor selumetinib (24). NHEK cells and HaCaT cells were treated with sorafenib singly and in combination with selumetinib and then UV irradiated. Sorafenib suppressed UV-induced apoptosis strongly, regardless of whether selumetinib was present (Fig. 2A and B). Selumetinib did not affect sorafenib-mediated suppression of apoptosis or JNK activation (lanes 5–8; Fig. 2C and D) despite potent abrogation of ERK phosphorylation (lanes 3–4, 7–8; Fig. 2C and D).

Multiple upstream kinases of JNK are inhibited by sorafenib

We then sought to determine how sorafenib suppresses JNK activation and apoptosis. Given that MKK4 and MKK7 activation were also suppressed at 1 μmol/L
sorafenib (Fig. 1C and D), we reasoned that sorafenib must affect upstream kinases. Comprehensive kinase profiling of sorafenib has been reported using a quantitative binding assay platform (25, 26); therefore, we performed in vitro kinase assays against a panel of 38 kinases reported to be upstream of JNK (21, 27) and other kinases previously tested against sorafenib using a 10-dose, 3-fold serial dilution starting at 10 μmol/L (Supplementary Table S1). None of the JNKs is directly inhibited.

We identified ZAK, TAO1/TAOK2, MAPK14 (p38α), and MAPK11 (p38β) as having low in vitro IC₅₀ values below 500 nmol/L (Supplementary Table S1). Using p38 inhibitors BIRB796 and LY2228820, we were unable to observe any suppression of UV-induced apoptosis (data not shown). Therefore, we focused on ZAK and TAOK2, which have the lowest biochemical IC₅₀ values of 48.6 and 59.8 nmol/L, respectively (Fig. 3A). These IC₅₀ values are in the same nanomolar range as those previously reported for BRAF and CRAF (2) and in our panel of assays (Supplementary Table S1).

ZAK is critically important for JNK activation upstream of MKK4 and MKK7 (28) and is important for doxorubicin-induced apoptosis (29, 30). TAOK2 has been implicated as an activator of JNK as well (31–35). To examine the requirements for ZAK and TAOK2 in promoting JNK activation and apoptosis more directly, we carried out lentiviral shRNA knockdown experiments in HaCaT cells. HaCaT cells with more than 85% knockdown of ZAK by quantitative real-time PCR (qRT-PCR, TaqMan; Fig. 3B) were created using two lentiviral shRNA clones (pGIPZ; Thermo), ZAK-1 and ZAK-4. Cells expressing shRNA ZAK-1 were then infected to express shRNA against TAOK2 as well (Fig. 3B). Both ZAK single knockdown and ZAK/TAOK2 double knockdown cells lack detectable ZAK protein expression and ZAK/TAOK2 double knockdown cells showed 91% reduction in TAOK2 mRNA (TaqMan; Fig. 3B). Cells expressing shRNA ZAK-1 and ZAK-4 were substantially more resistant than scramble shRNA-expressing (SCR) cells to UV-induced apoptosis (Fig. 3C). Knockdown of ZAK alone essentially accounted for the entire effect of sorafenib on UV-induced apoptosis (Fig. 3C). ZAK-1-shRNA–expressing HaCaT cells showed statistically significant, but small differences in suppression of apoptosis as compared with ZAK/TAOK2 double knockdown (12%) and sorafenib-treated SCR cells (17%; Fig. 3C). Importantly, JNK activation was substantially suppressed in ZAK-1-shRNA and ZAK/TAOK2 double knockdown cells at 1 and 6 hours after irradiation (Fig. 3D). The suppression is most evident by treatment with 1 μmol/L sorafenib (lanes 6, Fig. 3D), but it is clear that both single ZAK knockdown and double ZAK/TAOK2 knockdown both result in significant suppression of phospho-JNK (lanes 7 and 8, Fig. 3D).

Sorafenib suppresses JNK activation and apoptosis in vivo

To address whether sorafenib-mediated suppression of JNK signaling and apoptosis occurs in vivo, we...
examined cSCC arising in patients treated with sorafenib and compared them with sporadic cSCC that were histologically similar, arising in individuals never treated with sorafenib (Fig. 4A and B). Phospho-JNK (Fig. 4A) and cleaved caspase-3 (Fig. 4B) expression were probed using immunohistochemistry and quantified following normalization by unit area (mm$^2$) of tumor tissue (malignant keratinocytes) only (Fig. 4C). Sporadic cSCC arising in patients never treated with sorafenib ($n = 9$) had substantially greater expression of phospho-JNK ($P = 0.0045$; Fig. 4A and C) and cleaved caspase-3 ($P = 0.003$; Fig. 4B and C) as compared with lesions arising in sorafenib-treated patients ($n = 13$; Fig. 4A–C). Therefore, there are significant reductions in phospho-JNK (46%) and cleaved caspase-3 expression (88%) in human cSCC, showing that suppression of JNK activity and apoptosis occur in vivo in patients treated with sorafenib.
To model the emergence of UV-driven cSCC under controlled conditions, we exposed two cohorts of SKH1-E hairless mice to chronic low-dose (12.5 kJ/m² weekly) UV using solar simulators (Oriel). In this model of UV-driven cSCC development, we irradiated the mice for 72 days before initiation of drug (50 mg/kg daily, oral gavage) or control vehicle treatment. Papillomas were observed within 30 days of drug administration (Fig. 5A) and were histologically well differentiated (Fig. 5B). Some progressed to invasive cSCC and although the kinetics of lesion development were similar, drug-treated animals had 2.3-fold more lesions (P < 0.001; Fig. 5C), with no significant differences in the average size of lesions (2.77 ± 0.26 mm in diameter in controls vs. 3.00 ± 0.20 mm sorafenib-treated; P = 0.49). When we quantified the effects of sorafenib on JNK activation and apoptosis in these papillomas and cSCC, we found decreases in both phospho-JNK expression (51%; P = 0.037; n = 11 pairs) and cleaved caspase-3 expression (72%; P = 0.001; n = 11 pairs; Fig. 5D) in sorafenib-treated mice as compared with control-treated mice.

Paradoxical ERK activation is separable from JNK activity suppression

Although the effects of sorafenib on JNK-dependent apoptosis is clear and independent of ERK activity (Fig. 2), the relative contribution of paradoxical ERK activation versus JNK pathway inhibition to tumorigenesis has not been precisely addressed. To study this, we used the fact that paradoxical ERK activation requires CRAF (11–13). We used isogenic, matched WT and Craf⁻/⁻ MEFs and transformed them with adenovirus E1A and human HRASG12V to enable anchorage-independent growth. WT and matched Craf⁻/⁻ MEFs were plated in soft agar assays (16) and treated with sorafenib. Both WT and Craf⁻/⁻ MEFs exhibited a significant colony formation advantage in the presence of sorafenib (compare Fig. 6A with B). When normalized to untreated transformed WT and Craf⁻/⁻ MEFs, sorafenib treatment resulted in a 1.92-fold expansion of colony numbers in WT MEFs and a 1.66-fold expansion in Craf⁻/⁻ MEFs. On the basis of this analysis, we estimated that the effect of paradoxical ERK activation to be 13.5% and other effects, including inhibition of JNK activity, to account for the rest (86.5%) of the total colony growth advantage (Fig. 6C).

Figure 4. Sorafenib suppresses apoptosis and JNK signaling in vivo. A and B, cSCC samples from sorafenib-treated patients and nontreated patients (sporadic, first row) were analyzed by immunohistochemistry for phospho-JNK and cleaved caspase-3 expression. cSCC arising in sorafenib-treated patients (A and B, second row) show decreased expression of phospho-JNK (A) and cleaved caspase-3 (B) as compared with sporadic cSCC in patients never treated with sorafenib (second row). Scale bar, 100 μm. C, comparisons of stained cells normalized to mm² of tumor area revealed significant suppression of both phospho-JNK and cleaved caspase-3 expression in cSCC arising in sorafenib-treated patients (*, P < 0.01).

Figure 5. Sorafenib increases cSCC development in the UV-driven hairless mouse model. A and C, chronically irradiated SKH-1E hairless mice were treated with sorafenib (n = 5, SOR) and vehicle (n = 5, CON) starting at day 72. Tumors were induced within 30 days of sorafenib treatment and drug-treated (SOR) mice had significantly greater numbers of lesions than controls (CON; A, day 138). Papillomas were histologically well differentiated (B). Although tumors exhibited similar kinetics, there were 2.3-fold more tumors in sorafenib-treated mice as compared with vehicle-treated mice on average (C: ***, P < 0.001). D, tumors from sorafenib-treated animals showed lower levels of phospho-JNK (P = 0.037; *; P < 0.05) and cleaved caspase-3 (P = 0.001; **; P < 0.01) as compared with control-treated animals (n = 6 pairs).
Discussion

Compared with other RAF inhibitors such as vemurafenib, the incidence of cSCC associated with sorafenib is relatively low; however, this association is well established in multiple trials (4–6, 8–10). Although these proliferations are indolent and often easily treated, we further explored the mechanism of development of these lesions to make the broader point that adverse reactions are an opportunity to study how off-target pathways may result in biologically significant effects. As the use of sorafenib continues to expand, a thorough understanding of these effects is important in knowing how effects on nontarget tissues occur.

The selectivity of kinase inhibitors remains a crude estimate of the entire spectrum of potential biologic effects. Although sorafenib is not generally regarded as being highly selective, its inhibition of several kinases relevant for cancer cell signaling likely contributes to its clinical efficacy in vivo (1), and it is still unclear to what extent individual targeted kinases contribute to the observed efficacy in certain settings (36). What has remained relatively underexplored in the study of targeted inhibitors is how unintended targets such as ZAK affect the biology of nontarget cells (keratinocytes).

The prevailing explanation for why cSCC development is accelerated in RAF inhibitor–treated patients is paradoxical ERK activation, which occur most prominently in cells with mutant RAS. RAS mutations occur, along with mutations in TGFβRI and TP53, in cSCC arising in sorafenib-treated patients (4, 5); however, RAS mutations do not seem to be as overrepresented as they are in vemurafenib-induced lesions (16, 17), perhaps suggesting that the relative contribution of paradoxical ERK activation may be lower in sorafenib-exposed cells. Furthermore, paradoxical ERK activation seems to be short-lived (5). Therefore, these findings also suggest that other mechanisms play a role in accelerating the development of cSCC.

Because these cSCC arise relatively quickly and many of the lesions are on sun-exposed areas (4, 6), we specifically chose to look at UV exposure as a predisposing factor, because it is the predominant environmental risk factor for skin cancer. In the course of our studies, we examined multiple signaling pathways, including ERK, p38, and JNK and focused on the latter because of its demonstrated and critical role of UV-induced apoptosis (20, 37).

We have discovered a novel effect of sorafenib in inhibiting UV-induced JNK activation and apoptosis. This effect is independent of ERK signaling, and is not the result of paradoxical ERK activation observed with RAF inhibitors. After profiling several kinases upstream of JNK and p38, we showed that TAO2 and ZAK were the most likely targets and that inhibition of ZAK is predominantly responsible for this effect. Sorafenib has been previously shown to suppress ZAK and downstream JNK signaling (28–30, 38). Indeed, multiple inhibitors are known to inhibit ZAK, including nilotinib (30), suggesting it may be a promiscuous target of multiple small molecule kinase inhibitors. Whether this is due to structural similarities is not currently known.

We show for the first time that sorafenib suppresses JNK-dependent UV-induced apoptosis in cells and in epidermal squamous proliferations of both treated patients and mice. In addition, sorafenib-induced suppression of JNK signaling contributes to a significant portion of the acceleration of soft agar colony formation in vitro. cSCC arising in patients treated with sorafenib have substantially less phospho-JNK expression and apoptosis than sporadic cSCC arising in patients never treated with sorafenib (Fig. 4). Similarly, in our mouse model of UV-driven cSCC, both phospho-JNK expression and cleaved caspase-3 expression are inhibited by sorafenib (Fig. 5). Finally, we used a soft agar colony assay to test the relative contributions of paradoxical ERK activation and JNK-mediated apoptosis by using Craf−/−MEFs, which

**Figure 6.** Paradoxical ERK activation and JNK pathway inhibition make significant and separable contributions to sorafenib-induced growth. A and B, we envision two separable, parallel mechanisms by which sorafenib contributes to cSCC development. Drug-induced paradoxical ERK activation and inhibition of JNK signaling occur in parallel, but the former depends on intact CRAF. A and B, representative soft agar colonies of E1A and HRASG12V-transformed wild-type (WT; first row) and Craf−/− (second row) MEFs, following exposure to 1.0 μmol/L sorafenib (B) more than 4 to 6 weeks show significant colony-forming advantages conferred by sorafenib. C, the fold-change in colony counts of transformed WT (n = 36) and Craf−/− (n = 22) MEFs demonstrate a drug-induced increase in colonies, for both WT (1.92-fold) and Craf−/− (1.66-fold) MEFs. The difference between colony formation advantages conferred by 1.0 μmol/L sorafenib in WT versus Craf−/− MEFs was interpreted to reflect the contribution of paradoxical ERK signaling (red arrow), which depends upon Craf, and is 13.5% of the total effect reflected in the first two columns. The remainder of the total reaction is composed of other effects, including JNK inhibition (blue arrow). All differences within each MEF population were significant (**, P < 0.02; ***, P < 0.01).
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