The Multikinase Inhibitor Sorafenib Potentiates TRAIL Lethality in Human Leukemia Cells in Association with Mcl-1 and cFLIP\textsubscript{L} Down-regulation

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Abstract

Interactions between the multikinase inhibitor sorafenib and tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) were examined in malignant hematopoietic cells. Pretreatment (24 h) of U937 leukemia cells with 7.5 \textmu mol/L sorafenib dramatically increased apoptosis induced by sublethal concentrations of TRAIL/Apo2L (75 ng/mL). Similar interactions were observed in Raji, Jurkat, Karpas, K562, U266 cells, primary acute myelogenous leukemia blasts, but not in normal CD34\textsuperscript{+} bone marrow cells. Sorafenib/TRAIL–induced cell death was accompanied by mitochondrial injury and release of cytochrome c, Smac, and AIF into the cytosol and caspase-9, caspase-3, caspase-7, and caspase-8 activation. Sorafenib pretreatment down-regulated Bcl-xL and abrogated Mcl-1 expression, whereas addition of TRAIL sharply increased Bid activation, conformational change of Bak (ccBak) and Bax (ccBax), and Bax translocation. Ectopic Mcl-1 expression significantly attenuated sorafenib/TRAIL–mediated lethality and dramatically reduced ccBak while minimally affecting levels of ccBax. Similarly, inhibition of the receptor-mediated apoptotic cascade with a caspase-8 dominant-negative mutant significantly blocked sorafenib/TRAIL–induced lethality but not Mcl-1 down-regulation or Bak/Bax conformational change, indicating that TRAIL–mediated receptor pathway activation is required for maximal lethality. Sorafenib/TRAIL did not increase expression of DR4/DR5, or recruitment of procaspase-8 or FADD to the death-inducing signaling complex (DISC), but strikingly increased DISC–associated procaspase-8 activation. Sorafenib also down-regulated cFLIP\textsubscript{L}, most likely through a translational mechanism, in association with diminished eIF4E phosphorylation, whereas ectopic expression of cFLIP\textsubscript{L} significantly reduced sorafenib/TRAIL lethality. Together, these results suggest that in human leukemia cells, sorafenib potentiates TRAIL–induced lethality by down-regulating Mcl-1 and cFLIP\textsubscript{L}, events that cooperate to engage the intrinsic and extrinsic apoptotic cascades, culminating in pronounced mitochondrial injury and apoptosis. [Cancer Res 2007;67(19):9490–500]

Introduction

Sorafenib ( Nexavar, BAY43-9006) was initially identified as a Raf1 kinase inhibitor by high-throughput screening, although subsequent studies showed that it has additional activity against the serine/threonine kinases B-Raf, B-Raf(V600E); p38 mitogen-activated protein kinase; the receptor tyrosine kinases c-kit, Flt3, RET; and the proangiogenic receptor kinases vascular endothelial growth factor receptors 1, 2, 3; and platelet-derived growth factor receptor (reviewed in ref. 1). Sorafenib was recently approved by the Food and Drug Administration for treatment of advanced renal cell carcinoma (2). Through these actions, sorafenib blocks neoplastic cell signaling pathways, particularly those involved in growth and vascularization (3, 4) and avoidance of cell death (3, 5–7). Furthermore, we and others (3, 5, 6) recently showed that sorafenib potently down-regulates the antiapoptotic protein Mcl-1, a multidomain member of the Bcl-2 family implicated in malignant hematopoietic cell survival (8). In human leukemia cells, sorafenib-mediated Mcl-1 down-regulation is known to play a major functional role in lethality (5, 6). Notably, sorafenib-mediated Mcl-1 down-regulation stems from translation inhibition associated with diminished phosphorylation of the translation initiation factor elf4E (5). The ability of sorafenib to down-regulate Mcl-1 and inactivate elf4E has been confirmed in vivo in a human hepatocellular carcinoma tumor xenograft model (3).

Interactions between sorafenib and other targeted agents have been examined in a limited number of preclinical studies. For example, interactions between sorafenib and the proteasome inhibitor bortezomib (9) or the PKC\textsubscript{y} inhibitor rottlerin (10) have been observed in diverse preclinical settings, including epithelial neoplasms and leukemias. In several cases, enhanced cell death was associated with inactivation of prosurvival pathways such as Raf/extracellular signal-regulated kinase 1/2 and AKT (9, 10).

The ability of sorafenib to reduce neoplastic cell Mcl-1 expression (3, 5, 6) raises the possibility that it might be particularly effective in combination with agents whose actions are regulated by this protein. In this context, several recent studies have shown that Mcl-1 is a major modulator of tumor necrosis factor (TNF)–related apoptosis-inducing ligand (TRAIL)–induced lethality (11–13), suggesting that this agent might be a candidate for such a combination strategy. TRAIL is a member of the TNF family, which includes Fas-ligand (Fas-L) and TNF, each of which induces cell death through the receptor–mediated apoptotic pathway (14). In contrast to Fas-L and TNF, TRAIL induces apoptosis in diverse tumor cell types and xenographs while exhibiting little toxicity toward normal cells (14). TRAIL binds to five receptors: four of which are membrane-bound and one is a soluble receptor (reviewed in ref. 15). Two of these receptors, death receptors 4 (DR4) and 5 (DR5), are agonistic receptors containing a...
cytoplasmic death domain activated by TRAIL. Two other receptors, decoy receptors 1 (DcR1) and 2 (DcR2) act as antagonistic receptors. Upon binding of TRAIL homotrimer to DcR4 or DcR5, which induces trimerization of the receptors, a death-inducing signaling complex (DISC) is formed. The adaptor protein FADD is then recruited to the complex, facilitating incorporation of the initiator procaspase-8 prodomain into the DISC. This leads to activation of procaspase-8, a potent activator of downstream effector caspases (e.g., caspase-3, caspase-6, and caspase-7; ref. 15). The death receptor–initiated apoptotic pathway is referred to as the extrinsic apoptotic pathway, in contrast to the intrinsic/mitochondrial pathway, which involves mitochondrial injury, release of proapoptotic proteins into the cytosol (e.g., cytochrome c), and activation of procaspase-9 (16). Cross-talk occurs between the intrinsic and extrinsic pathways through Bid, a BH3–only protein member of the Bcl-2 superfamily that is activated by caspase-8 and triggers mitochondrial injury (8). When the intrinsic pathway is activated, multidomain proapoptotic members of the Bcl-2 gene family (e.g., Bax, which translocates to the mitochondria, and Bak, which undergoes a conformational change; ref. 17), induce release of cytochrome c and other mitochondrial factors into the cytosol, leading to formation of the apoptosome and activation of caspase-9 and caspase-3 (15). Simultaneous activation of the intrinsic and extrinsic apoptotic pathways leads to mutual amplification and a marked potentiation of apoptosis (18, 19).

The present findings reveal that preexposure of human leukemia cells to sorafenib dramatically sensitize them to the lethal effects of TRAIL and, as hypothesized, sorafenib-mediated Mcl-1 down-regulation plays a significant functional role in these interactions. They also show that down-regulation of the short-lived protein and procaspase-8 regulatory molecule cFLIP, primarily through translation inhibition, contributes significantly to sorafenib/TRAIL synergism. Specifically, sorafenib-mediated cFLIP down-regulation triggers a pronounced engagement of the DISC and procaspase-8 processing, resulting in the striking generation of truncated Bid (tBid). Collectively, these findings suggest that in human leukemia cells, sorafenib-mediated down-regulations of Mcl-1 and cFLIP cooperate to activate both the intrinsic and extrinsic cell death pathways, triggering a dramatic increase in leukemia cell death.

**Materials and Methods**

**Cells and cell culture.** U937 human leukemia cells were obtained from American Type Culture Collection, and cultured and maintained as described previously (20). U937 cells stably expressing either Mcl-1 or dominant-negative caspase-8 or their empty vector counterparts were obtained as reported previously (18, 21). U937 cells expressing cFLIP were generated by cloning a cDNA containing the entire c-FLIP coding region (CFLAR, Genbank accession no. NM_003879) in pcDNA3.1/V5-His-TOPO vector (Invitrogen). All experiments used cells in logarithmic phase at 2.5 × 10^6/mL. Acute myelogenous leukemia (AML) blasts were obtained with informed consent from patients with AML undergoing routine diagnostic aspirations with approval from the Virginia Commonwealth University Institutional Review Board. Informed consent was provided according to the Declaration of Helsinki. AML blasts were isolated and cultured as described previously (21). Normal mononuclear cells were also obtained with informed consent from the bone marrow of patients with nonmalignant hematopoietic disorders (e.g., iron deficiency anemia, immune thrombocytopenia). Mononuclear cell preparations were obtained as described for the isolation of AML blasts. CD34^+ cells were purchased from Cambrex.

**Drugs and chemicals.** Sorafenib (BAY43-9006, Bayer) was provided by the Cancer Treatment and Evaluation Program, National Cancer Institute, NIH, dissolved in DMSO, and aliquots maintained at −80°C. TRAIL/Apo2L was purchased from Alexis and stored in aliquots at −80°C. The pan-caspase inhibitor BOC-D-fms was purchased from Enzyme System Products and dissolved in DMSO.

**Assessment of apoptosis.** Apoptotic cells were evaluated by Annexin V/propidium iodide (BD Pharmingen) staining as per the manufacturer's instructions as previously described (21) and by morphologic assessment of Wright-Giemsa–stained cytosin preparations.

**Assessment of mitochondrial membrane potential (ΔΨm).** After treatment, cells were harvested and 2 × 10^5 incubated with 40 nmol/L DiOC6 (15 min, 37°C). Loss of mitochondrial membrane potential was determined by flow cytometry as previously described (22).

**Analysis of cytosolic cytochrome c and AIF.** A previously described technique was used to isolate the S-100 (cytosolic) cell fraction of treated cells (22). For each condition, 30 mg of protein isolated from the S-100 cell fraction were separated and detected by Western blot.

**Western blot analysis.** Whole cell pellets were washed and resuspended in PBS, and lysed with loading buffer (Invitrogen) as previously described (22). Thirty micrograms of total protein for each condition were separated by 4% to 12% Bis-Tris NuPage precast gel system (Invitrogen) and electrobolted to nitrocellulose. After incubation with the corresponding primary and secondary antibodies, blots were developed by enhanced chemiluminescence (New England Nuclear).

**Antibodies for Western blot analysis.** Primary antibodies for the following proteins were used at the designated dilutions: poly(ADP-ribose) polymerase (PARP; 1:1,000; BioMol); Mcl-1; procaspase-3; cytochrome c; XIAP (1:1,000; BD Pharmingen); caspase-8 (1:2,000, Alexa Fluor); Bid (1:1,000, Cell Signaling); phosphorylated eIF4E (1:500, Cell Signaling); eIF4E; cFLIP (1:500, Santa Cruz Biotechnology); anti-His tag (1:500, Serotec); AIF (1:1,000, Santa Cruz Biotechnology); actin (1:4,000; Sigma-Aldrich); DR4, DR5 (1:500, Imgenex). Secondary antibodies conjugated to horseradish peroxidase were obtained from Kirkegaard and Perry Laboratories, Inc.

**Real-time reverse transcription-PCR.** Real-time reverse transcription-PCR (RT-PCR) was done in triplicate using the SensiMix One-Step SYBR green solution (Bioline) and cFLAR QuantiTec Primer Assay (Qiagen). Results for the experimental gene were normalized to 18S rRNA levels.

**Determination of caspase-3/7 and caspase-9 activity.** Treated cells were lysed, and equivalent quantities were assayed according to the manufacturer's instructions (caspase-3/7 and caspase-9 assay kits; BioVision). The fold increase in activity was calculated as the ratio between values obtained for treated samples versus those obtained in untreated controls.

**Isolation of the TRAIL DISC.** DISC precipitation was done by using an anti-His tag antibody. 6×His-tagged recombinant TRAIL (Alexis) was preincubated for 15 min with the primary anti-His antibody, after which cells were treated for the times indicated in the corresponding figures. Control U937 cells or cells exposed to sorafenib alone were incubated with the primary anti-His tag antibody. DISC formation was then terminated, and cells were washed thrice in ice-cold PBS. Cells were lysed in radioimmunoprecipitation assay buffer (1% NP40, 0.5% sodium deoxycolate, 0.1% SDS, in PBS) containing Complete protease inhibitors (Roche Molecular Biochemicals) for 30 min on ice followed by centrifugation at 15,000 × g for 10 min at 4°C. The TRAIL DISC was then precipitated using anti-mouse–conjugated Dynabeads (Invitrogen) at 4°C overnight. Precipitates were washed six times with lysis buffer, and receptor complexes were eluted with 30 µL of sample buffer followed by Western blotting analysis.

**Bax/Bak conformational change.** To analyze conformational change of Bax and Bak, cells were lysed in CHAPS lysis buffer and immunoprecipitated in lysis buffer by using 500 µg of total cell lysate and 2.5 µg of either anti-Bax 6A7 (Sigma-Aldrich) or anti–Bak-Ab1 (Calbiochem) antibodies that recognize conformationally changed Bax and Bak, respectively. Resulting immune complexes were analyzed by Western blot and probed with an anti-Bax or anti-Bak antisem (Santa Cruz Biotechnology).

**Statistical analysis.** The significance of differences between experimental conditions was determined using the Student’s t test for unpaired observations. To assess drug interaction, median dose analysis (23) was...
The combination index (CI) was calculated for a two-drug combination involving a fixed concentration ratio. CI values <1.0 indicate a synergistic interaction.

**Results**

Preexposure to sorafenib potently increases TRAIL-induced lethality in human leukemia cells. Interactions between sorafenib and TRAIL were first investigated in U937 human leukemia cells. To this end, various administration sequences were tested, including coadministration or preexposure (24 h) to one of the agents followed by addition of the second agent. For these studies, TRAIL was administered at multiple concentrations, including 10, 25, 50, 75, and 100 ng/mL, and sorafenib was administered at concentrations of 5 or 7.5 μmol/L, which were minimally or only modestly toxic alone (Fig. 1A). Although either simultaneous exposure to sorafenib/TRAIL (24 h) or preexposure to TRAIL followed by sorafenib (T24 h S24 h) resulted in modest activity (e.g., f45–50% lethality; data not shown), 24 h exposure to sorafenib followed by addition of TRAIL concentrations ≥50 ng/mL (i.e., 50, 75, 100 ng/mL) markedly increased cell death, ranging from 60% for 5 μmol/L sorafenib to >80% to 90% in the case of 7.5 μmol/L sorafenib (Fig. 1A). Consequently, for all subsequent studies, the optimal sequence of sorafenib (7.5 μmol/L) followed by TRAIL (75 ng/mL) was investigated.

**Figure 1.** Preexposure to sorafenib potently increases TRAIL-induced lethality in human leukemia cells. A, U937 cells were sequentially exposed to sorafenib (S; 5 and 7.5 μmol/L) for 24 h after which TRAIL (T; 7, 10, 25, 50, 75, and 100 ng/mL) was added. The extent of apoptosis was then determined by Annexin V/propidium iodide analysis as described in Materials and Methods. B, time course analysis of sorafenib/TRAIL–induced cell death (left and right) and loss of mitochondrial membrane potential (Δψm, center and right). U937 cells were preexposed to sorafenib for 24 h (S24) after which TRAIL (75 ng/mL) was added; cells were collected at the indicated time intervals and evaluated for both cell death and loss of Δψm by flow cytometry as described in Materials and Methods. Columns, means for three separate experiments; bars, SE. *, significantly higher than percentages observed with any drug administered alone (A and B); #, significantly higher than percentages of cell death (B, right); in both cases, P < 0.01. C, mitochondrial proteins (cytochrome c, Smac, AIF) were determined by Western blot in the S100 fraction whereas whole lysates were used for caspase-8, caspase-3, and PARP analysis. In all cases, 30 μg of proteins were separated by SDS-PAGE as described in Materials and Methods and probed with the indicated antibodies; blots were probed with antibodies directed against actin to ensure equivalent loading and transfer. Representative blots are shown. Two additional experiments gave similar results. D, median dose-effect analysis of apoptosis induction in U937 cells sequentially exposed 24 h to sorafenib followed by TRAIL (24 h) administered over a range of concentrations at a ratio of 1:10. CI values for each fraction affected <1.0 correspond to synergistic interactions.
Detailed time course analysis of sorafenib (7.5 μmol/L)/TRAIL (75 ng/mL)–induced lethality revealed the rapid onset of cell death as early as 2 h after TRAIL addition, and which was very extensive by 8 h (Fig. 1B, left). Increased cell death correlated closely with a pronounced increase in mitochondrial injury: that is, loss of mitochondrial membrane potential (ΔΨm; Fig. 1B, center), an event that preceded the induction of apoptosis (Fig. 1B, right), as well as release of the proapoptotic mitochondrial proteins cytochrome c, Smac/DIABLO, and AIF into the cytosol (Fig. 1C). These events were accompanied by activation/cleavage of procaspase-8 as well as procaspase-3, procaspase-7, and procaspase-9 (Fig. 1C; Supplementary Fig. S1), and degradation of PARP (Fig. 1C). Consistent with these findings, sequential exposure of U937 to sorafenib/TRAIL in the presence of the pan-caspase inhibitor BOC-D-fmk (20 μmol/L) significantly diminished the extent of cell death (P < 0.01; Supplementary Fig. S2), demonstrating the caspase dependence of this interaction. Median dose-effect analysis of < 0.01; Supplementary Fig. S2), demonstrating the caspase

Sequential exposure to sorafenib and TRAIL leads to the marked down-regulation of Mcl-1, increased levels of tBid, conformational change in Bax/Bak, and Bax translocation. To identify mechanisms responsible for synergistic interactions between sorafenib and TRAIL, expression patterns of several proapoptotic and antiapoptotic proteins were investigated. As shown in Fig. 2A, no changes were observed in total levels of Bcl-2 and survivin after combined exposure of U937 cells to sorafenib and TRAIL, whereas a moderate reduction in Bcl-xl expression and the appearance of an XIAP cleavage fragment were observed. As previously reported (5), sorafenib markedly reduced Mcl-1 protein levels, which was almost absent when TRAIL was administered (S24; Fig. 2A). Furthermore, in sorafenib-pretreated cells, TRAIL exposure induced a pronounced reduction in expression of the BHS domain–only protein Bid, which is cleaved by active caspase-8 (24), and a concomitant increase in expression of tBid (Fig. 2A). Although Bak or Bax total expression did not change with any treatment, a dramatic increase in the expression of the active, conformationally changed form of these proteins (25, 26) was noted in sorafenib-pretreated cells exposed to TRAIL (Fig. 2B and C). Bak and Bax conformational change appeared as early as 2 h after addition of TRAIL to sorafenib-pretreated cells, and increased progressively over the ensuing 6 h. Moreover, treatment of cells individually with sorafenib (24 or 32 h) or TRAIL (8 h) minimally affected or induced a modest change, respectively, in levels

**Figure 2.** Antiapoptotic and proapoptotic proteins in sorafenib/TRAIL–treated U937 cells. A, U937 cells were exposed to sorafenib (7.5 μg), TRAIL (75 ng/mL), or their combination for the indicated time intervals, and at the end cells were pelleted, lysed, and 30 μg of protein were separated by SDS-PAGE. Blots were probed with the corresponding antibodies and subsequently stripped and probed with antibodies directed against actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. cf, cleavage fragment. B and C, analysis of Bak/Bax conformational change; after treatment as described in A, conformationally changed Bak and Bax were determined by first immunoprecipitating lysates with an anti–Bak-Ab1 or anti–Bax-6A7 antibodies, which recognize only the conformationally changed protein, followed by immunoblotting with an anti-Bak or an anti-Bax antibody, respectively, as described in Materials and Methods. Each lane was loaded with 30 μg of protein; IgG controls confirm equivalent loading and transfer. C, bottom, translocation of Bax was analyzed by monitoring expression of the protein in the cytosolic and pelleted fractions as described in Materials and Methods. The results of a representative study are shown; two additional experiments yielded similar results. IP, immunoprecipitation; WB, Western blot.
of cytosolic Bax (Fig. 2C). However, sequential administration (S24 h→T) induced a marked redistribution of Bax from the cytosolic to the particulate (mitochondrial) compartment within 2 to 4 h of addition of TRAIL to sorafenib-pretreated cells (Fig. 2C). Thus, sequential exposure of leukemia cells to sorafenib followed by TRAIL induced a striking decline in expression of Mcl-1, Bid cleavage and increased levels of tBid, and a pronounced Bak/Bax conformational change and Bak translocation.

**Sorafenib-induced Mcl-1 down-regulation plays a significant functional role in potentiation of TRAIL lethality.** Mcl-1 is a potent antiapoptotic member of the Bcl-2 family (8). To define the functional role of Mcl-1 down-regulation in sorafenib/TRAIL lethality, U937 cells ectopically expressing Mcl-1 (21) were used. Ectopic Mcl-1 expression partially but significantly reduced sorafenib/TRAIL-induced cell death (Fig. 3A) and loss of ΔΨm (Fig. 3B) compared with U937/empty vector cells (P < 0.005 in each case). The protection noted in sorafenib/TRAIL–treated U937/Mcl-1 cells was compatible with the observation that although Mcl-1 down-regulation (±TRAIL) clearly reduced expression of ectopic Mcl-1 in U937/Mcl-1 cells, residual Mcl-1 expression levels were substantially greater than those of U937/empty vector cells treated in a similar manner (Fig. 3C). Recent studies suggest that Mcl-1 antiapoptotic activity is related to its association with and inactivation of Bak (27), prompting investigation of the effects of ectopic Mcl-1 expression on the regulation of Bak activity/conformational change. Notably, empty vector U937 cells pretreated with sorafenib displayed a rapid increase in conformationally changed Bak after TRAIL administration. A similar pattern was observed with Bax (Fig. 3C). In contrast, U937/Mcl-1 cells exhibited essentially no Bak conformational change and a more modest but discernible reduction in Bax conformational change/activation. These results suggest that sorafenib-induced Mcl-1 down-regulation plays a functional role in potentiating TRAIL-induced Bak and Bax conformational change and lethality, and that Bak activation may be a particularly important contributor to synergism.

**Figure 3. Ectopic expression of Mcl-1 protects cells against sorafenib/TRAIL–induced cell death.** A and B, U937 cells expressing either ectopic Mcl-1 (U/Mcl-1, clones 14 and 16) or their empty vector counterparts (U/EV) were treated with sorafenib (7.5 μmol/L) for 32 h, TRAIL, for 8 h (75 ng/mL), or their combination (S24→ST8 h), after which both cell death (A) and loss of mitochondrial membrane potential (B) were done as described in Materials and Methods. Columns, means for three separate experiments; bars, SE. *, *P < 0.01, significantly less than values for empty vector controls (for A and B). C, both U937/EV and U937/Mcl-1 cells were exposed to sorafenib (7.5 μg/L), TRAIL (75 ng/mL), or their combination for the indicated time intervals, and at the end cells were pelleted, lysed, and 30 μg of protein were separated by SDS-PAGE, blotted, and probed with the corresponding anti–Mcl-1 and actin (to ensure equivalent loading and transfer) antibodies. The results of a representative study are shown; two additional experiments yielded similar results. D, analysis of Bak/Bax conformational change. U937/EV and U937/Mcl-1 cells were exposed to sorafenib (7.5 μg/L), TRAIL (75 ng/mL), or their combination (S24→T) for the indicated time intervals; lysates were prepared and conformationally changed; Bak and Bax were determined by first immunoprecipitating lysates with an anti–Bak-Ab1 or anti–Bax-6A7 antibody, which recognize only the conformationally changed protein, followed by immunoblotting with anti-Bak or anti-Bax antibodies, respectively, as described in Materials and Methods. Each lane was loaded with 30 μg of protein; IgG controls confirm equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results.
that in addition to effects on Mcl-1, sorafenib may also modulate the receptor pathway to increase TRAIL sensitivity.

**Sequential exposure to sorafenib and TRAIL is associated with enhanced activation of the DISC but not DR4 or DR5 expression.** Previous evidence in various cell types, including leukemia cells, that certain agents increase TRAIL sensitivity by modulating components of the extrinsic pathway, including up-regulation of death receptors (28–31), prompted us to investigate whether this phenomenon might be involved in sorafenib/TRAIL synergism. However, analysis of DR4 and DR5 levels by Western blot (Supplementary Fig. S5A), or monitoring receptors by flow cytometry (Supplementary Fig. S5B), revealed no changes in total expression under any experimental conditions. As a positive control, Jurkat cells exposed to the histone deacetylase (HDAC) inhibitor NVP-LAQ824 were investigated in parallel and, as previously reported (32), displayed a clear increase, reflected by an increase in fluorescence intensity and shift to the right, in the number of DR5 receptors localized to the cell membrane (Supplementary Fig. S5B). Together, these findings argue against increased death receptor expression as a mechanism underlying sorafenib/TRAIL synergism interactions.

The effects of preexposure to sorafenib were then examined with respect to TRAIL-induced formation of the DISC (Fig. 5A). Comparisons were made between recruitment of procaspase-8 and FADD into the DISC by TRAIL, either administered alone (left) or following prior exposure of cells to sorafenib (right). First, sorafenib alone had no effect on recruitment of FADD or procaspase-8 to the DISC. Second, the amount of procaspase-8 and FADD recruited to the DISC after TRAIL exposure was similar in untreated versus sorafenib-pretreated cells. These findings indicate that sorafenib is unlikely to act by enhancing the ability of TRAIL to recruit these molecules to the DISC. However, whereas in control cells TRAIL failed to induce processing of procaspase-8 into its p43 and p41 forms, a well-described characteristic of DISC activation (30), in sorafenib-pretreated cells the appearance of the activated procaspase-8 cleavage fragments occurred early and was very pronounced (Fig. 5A, right). Collectively, these results indicate that preincubation with sorafenib has little or no effect on levels of death receptors or DISC formation in leukemia cells exposed to TRAIL, but instead strikingly potentiates processing/activation of procaspase-8 recruited to the DISC.

**Sorafenib preexposure facilitates DISC-associated procaspase-8 activation by down-regulating cFLIP.** In view of the preceding findings, attention then focused on the procaspase-8 inhibitor cFLIP (FLICE-inhibitory protein). As shown in Fig. 5B (left), exposure of U937 cells to sorafenib alone induced a marked and rapid decline in cFLIP levels, which was detectable after 4 h of exposure. When TRAIL was added to sorafenib-treated cells, although residual cFLIP expression was very low, a further decline could be discerned that was accompanied by the appearance of a

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**Figure 4.** Interference with the receptor apoptotic pathway blocks sorafenib/TRAIL–induced lethality. A, U937 cells expressing either ectopic procaspase-8 dominant-negative (U/casp8DN) or their empty vector counterparts (U/EV) were treated with sorafenib (7.5 μM/L) for 32 h, TRAIL for 8 h (75 ng/mL), or the combination (S<sub>24</sub>→ST<sub>75</sub>) after which both cell death (left) and loss of mitochondrial membrane potential (∆Ψ<sub>mit</sub>; right) were done as described in Materials and Methods. Columns, means for three separate experiments; bars, SE. *P < 0.01, significantly less than values for empty vector controls. B, Western blot analysis of protein lysates from U/casp8DN and U/EV; cells were treated with sorafenib (7.5 μM) for 24 h after which TRAIL (75 ng/mL) was added; cells were then collected at the indicated intervals and processed as described in Materials and Methods. Blots were probed with the corresponding antibodies and subsequently stripped and probed with antibodies directed against actin to ensure equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results. C, expression levels of Mcl-1 were determined in whole lysates of U/casp8DN and U/EV; cells exposed to sorafenib (7.5 μM/L), TRAIL (75 ng/mL), or the combination (S<sub>24</sub>→T) as indicated; blots were subsequently stripped and probed with an antiactin antibody to ensure equivalent loading and transfer. Analysis of conformational change in Bax and Bak was done using extracts from the same cells; lysates were prepared, immunoprecipitated with either anti-Bak–Ab1– or anti–Bax–6A7–specific antibodies, followed by immunoblotting with anti-Bak or anti-Bax antibodies, respectively, as described in Materials and Methods. Each lane was loaded with 30 μg of protein; IgG controls confirm equivalent loading and transfer. The results of a representative study are shown; two additional experiments yielded similar results.
low molecular weight species, presumably representing a cleavage fragment (Fig. 5B, right). No changes were detected in the 26-kDa short form of cFLIP, cFLIPs (Fig. 5B, bottom). To investigate the functional role of cFLIPL down-regulation in responses to this regimen, U937 cells were transiently transfected with a cDNA coding for cFLIPL containing a 6×His tag. As shown, enforced expression of cFLIPL significantly reduced sorafenib/TRAIL lethality (Fig. 5C, left; * P < 0.01 versus empty vector controls). Cells were also collected for Western blot analysis (right) of expression of cFLIP (detected by an anti-6×His tag), Mcl-1, and actin; a representative blot is shown. Levels of cFLIPL were also monitored in U937 cells ectopically expressing Mcl-1 (U937/Mcl-1). In these cells, sorafenib continued to induce a dramatic decline in cFLIPL expression (Fig. 5D), suggesting that perturbations in these proteins act independently and cooperatively to promote apoptosis in sorafenib/TRAIL–treated cells.

Sorafenib-mediated cFLIPL down-regulation involves a translational component. cFLIPL is a short-lived protein (33), and several mechanisms have been implicated in its regulation, including transcriptional modulation (34, 35) and ubiquitin- and caspase-mediated degradation (30, 36, 37). To determine whether cFLIPL down-regulation reflected secondary, caspase-dependent events, sorafenib/TRAIL–treated cells were coincubated with the pan-caspase inhibitor BOC-D-fmk (20 μmol/L). As shown in Fig. 6A (left), in the presence of BOC-D-fmk, the low molecular weight fragment previously described in Fig. 5B (cf) was almost completely eliminated, but the expression of cFLIPL was only minimally restored. These findings indicate that although a contribution of caspase-mediated degradation to the down-regulation of cFLIPL after TRAIL administration cannot be completely excluded, it is unlikely to represent the major mechanism of reduced FLIPL expression. Moreover, no changes in cFLIPL expression were observed in extracts obtained from cells coexposed to the proteasome inhibitor MG-163 (Fig. 6A, M, right), arguing against a posttranslational mechanism of cFLIPL down-regulation. Finally, levels of cFLIPL mRNA were determined by real-

Figure 5. Preexposure of U937 cells to sorafenib facilitates DISC-associated procaspase-8 activation by down-regulating cFLIP. A, analysis of DISC formation was done as described in Materials and Methods in U937 cells exposed to sorafenib (7.5 μg), TRAIL (75 ng/mL), or the combination (S24→T) for the indicated intervals; levels of component of the DISC (e.g., procaspase-8 and FADD) were analyzed by Western blotting. Data are representative of three independent experiments. B, levels of cFLIPs/L were determined by Western blot in samples from U937 cells exposed to sorafenib (7.5 μg), TRAIL (75 ng/mL), or the combination (S24→T); 30 μg of protein were separated by SDS-PAGE, blotted, and probed with the corresponding antibodies. The results of a representative study are shown; two additional experiments yielded similar results. C, U937 cells were transiently transfected with either the empty vector (U937/EV) or with a construct coding for full-length cFLIP. After transfection, they were exposed to sorafenib (7.5 μg), TRAIL (75 ng/mL), or their combination (S24→T) as indicated, and cell death was evaluated by Annexin V/propidium iodide staining (left). *, P < 0.01, significantly less than values obtained for cells transfected with empty vector controls. Cells were also collected for Western blot analysis (right) of expression of cFLIP (detected by an anti-6×His tag), Mcl-1, and actin; a representative blot is shown. D, levels of cFLIP were determined by Western blot analysis in U937/Mcl-1 cells as described in Materials and Methods; cells were treated with sorafenib and TRAIL as described above.
time RT-PCR (Fig. 6B). As shown, exposure to sorafenib alone (4–8 h) failed to reduce cFLIPL mRNA levels (P > 0.05 compared with controls), despite the virtually complete reduction in protein expression (Fig. 5B). At later intervals (e.g., 24–26 h), sorafenib only modestly reduced cFLIP mRNA levels, and this effect was not significantly enhanced by addition of TRAIL (P > 0.05 versus sorafenib alone). Together, these findings argue that inhibition of transcription is unlikely to play a major role in the observed reduction in cFLIP expression.

Previous studies showed that sorafenib-mediated Mcl-1 down-regulation primarily involved inhibition of translation (5), an event associated with the rapid dephosphorylation of the mRNA cap-binding protein eIF4E (3, 5). Notably, exposure of cells to sorafenib ± TRAIL induced a dramatic decline in phosphorylation of eIF4E without modifying protein levels (Fig. 6C). To further examine this question, U937 cells were exposed to the transcription inhibitor actinomycin D (5 μg/mL) in the presence or absence of sorafenib (Fig. 6D), as previously described in the case of studies involving Mcl-1 (5). Our hypothesis was that if sorafenib modified cFLIP levels primarily at the transcriptional level, combined exposure to actinomycin D and sorafenib would have little further effect on cFLIP down-regulation; conversely, simultaneous inhibition of both transcription and translation should have a greater effect on cFLIP levels than blocking transcription alone (i.e., by actinomycin D). As shown, coexposure of cells to sorafenib and actinomycin D reduced cFLIP protein levels by ~50% compared with the effects of actinomycin D alone. Collectively, these findings support the notion that as in the case of Mcl-1 (5), inhibition of translation plays a significant functional role in sorafenib-mediated cFLIP down-regulation.

Discussion

The rationale for the present studies stemmed from recent observations showing that, first, exposure of human leukemia cells to sorafenib induced apoptosis through a mechanism involving Mcl-1 down-regulation via inhibition of translation (5); and second, evidence that Mcl-1 plays an important role in regulating the sensitivity of cells to TRAIL-induced apoptosis (11–13). The results of the present study indicate that preexposing human leukemia cells to sorafenib markedly sensitizes them to TRAIL and, as hypothesized, sorafenib-mediated Mcl-1 down-regulation plays an important functional role in this interaction. However, the present findings also suggest that sorafenib-mediated Mcl-1 down-regulation is not solely responsible for potentiation of TRAIL lethality, and that additional factors, particularly down-regulation of cFLIP, represent important contributors to the antileukemic synergism of this regimen.

Mcl-1 is a BH-multidomain member of the Bcl-2 family that exhibits potent antiapoptotic activity (38) and plays a particularly important role in the survival of malignant hematopoietic cells (5, 32, 39). Mcl-1 modulates apoptosis through multiple mechanisms, including interactions with proapoptotic members of the Bcl-2 family such as BH3-only domain proteins, for example, tBid (11) and Bim (13), as well as multidomain members such as Bak (27). In this context, several recent studies have shown that Mcl-1 cooperates with Bcl-xL to maintain Bak in an inactive state and that interference with both of these antiapoptotic proteins is required for full engagement of the apoptotic cascade (27). Therefore, it may be significant that in addition to down-regulation of Mcl-1, sorafenib/TRAIL–treated cells also exhibited a partial but clearly discernible reduction in Bcl-xL expression (Fig. 2). The finding that enforced
expression of Mcl-1 significantly diminished sorafenib/TRAIL–induced Bak conformational change as well as lethality argues that down-regulation of Mcl-1 plays an important role in Bak activation and mitochondrial injury in cells exposed to this regimen.

Cooperation between activation of the intrinsic and extrinsic apoptotic pathways has been extensively described (reviewed in refs. 17, 40). Evidence that Mcl-1 plays a role in controlling apoptosis by binding active Bid (tBid; ref. 11) therefore provides a theoretical basis for the observed synergism between sorafenib and TRAIL. For example, in receptor-mediated induction of apoptosis, activation of Bid represents a critical component of the caspase-8, thereby functioning as caspase-8 dominant-negative inhibitors described, including cFLIPS (26 kDa), cFLIPR (a 24-kDa form that structurally related to procaspase-8 but lacking enzymatic activity of procaspase-8 is cFLIP, which is recruited along with procaspase-8 present within the DISC. At this level, the most potent inhibitor sorafenib/TRAIL–treated cells was activation of procaspase-8, raised the possibility that sorafenib might modify the observation that sorafenib/TRAIL lethality was dramatically reduced in U937 cells ectopically expressing dominant-negative procaspase-8, and that sorafenib-mediated Mcl-1 down-regulation potentiates TRAIL lethality by two separate but related mechanisms: reduction in Mcl-1 inhibitory effects on Bak and promotion of Bid activation and proapoptotic actions.

Certain human leukemia cells, such as U937 cells, are particularly resistant to TRAIL-induced apoptosis (18, 31) and are classified as type II; that is, receptor-mediated apoptosis requires engagement of the mitochondrial pathway for maximal efficiency. In contrast, in type I cells, activation of the extrinsic pathway is fully capable of triggering a robust apoptotic response (43). Previous findings that U937 cells exhibited only modest cell killing in response to concentrations of TRAIL up to 15-fold higher than those used in the present study (i.e., ~1 μg/ml; refs. 18, 31), and the observation that sorafenib/TRAIL lethality was dramatically reduced in U937 cells ectopically expressing dominant-negative procaspase-8, raised the possibility that sorafenib might modify the TRAIL/receptor cascade through events unrelated to Mcl-1. For example, it has been shown that cells normally resistant to TRAIL can be sensitized to this molecule by coadministrating agents that modulate TRAIL receptors, including up-regulation of TRAIL-R1 (DR4) and TRAIL-R2 (DR5). Such agents include HDAC inhibitors and various cytotoxic compounds (28, 29, 44). Alternatively, other agents may act by down-regulating decoy receptors TRAIL-R3 and TRAIL-R4 (45). In addition, modulation of antiapoptotic proteins such as XIAP (31), or down-regulation of the procaspase-8 inhibitor cFLIP (30), has been shown to enhance TRAIL sensitivity. Preexposure to sorafenib had no effect on either expression of death receptors or the formation of the DISC itself, arguing against a contribution of these factors to the observed antileukemic synergism. However, the most striking difference noted in sorafenib/TRAIL–treated cells was activation of procaspase-8 present within the DISC. At this level, the most potent inhibitor of procaspase-8 is cFLIP, which is recruited along with procaspase-8 and FADD to the DISC (46). cFLIP is a short-lived protein structurally related to procaspase-8 but lacking enzymatic activity (33, 46). As many as 11 distinct cFLIP splice variants have been described, including cFLIP_

Ongoing studies are currently addressing the possible role that sorafenib-induced ER stress may play in the lethal actions of the sorafenib/TRAIL regimen.
Taken together, the preceding findings suggest a theoretical model that might account for the observed interactions between sorafenib and TRAIL. According to this model (Supplementary Fig. S6), sorafenib acts largely via a translational mechanism to downregulate the key antiapoptotic proteins Mcl-1 and cFLIP L, events that cooperate to sensitize human leukemia cells to TRAIL. A corollary of this model is that certain human leukemia cells may be resistant to TRAIL due to increased expression of Mcl-1 and cFLIP L. By disrupting the translational machinery and reducing these expressions of these proteins, sorafenib promotes cooperation between the intrinsic and extrinsic apoptotic pathways. Specifically, by reducing Mcl-1 expression, sorafenib releases Bak from one of its major inhibitory proteins (27). However, elimination of Mcl-1 may be necessary but not sufficient to trigger apoptosis, and additional events might be required. Such signals are provided by TRAIL via activation of the receptor pathway and recruitment of procaspase-8 through the death domain–containing adaptor molecule FADD to the DISC. When cFLIP L, a potent inhibitor of procaspase-8, is down-regulated by sorafenib, procaspase-8 is rapidly and extensively cleaved, leading to activation of its effector substrates procaspase-3 and Bid. Finally, activated Bid (tBid) provides a mechanism for cross-talk between the receptor and the intrinsic pathways by interacting with both Bax as well as Bak, which has become unthetered as a consequence of Mcl-1 down-regulation. These events lead to Bak and Bax conformational change and Bak translocation to the outer mitochondrial membrane, culminating in oligomerization and permeabilization of the mitochondria and release of cytochrome c, AIF, and Smac into the cytosol (51). Thus, sorafenib acts through both the intrinsic and extrinsic arms of the apoptotic pathway to lower the threshold for TRAIL-induced lethality. The ultimate implications of these findings will depend on several factors, including whether these events occur in vivo and whether effective plasma concentrations of these agents can be achieved. In this context, steady-state plasma sorafenib concentrations in excess of those used in the present study (e.g., -10 μmol/L) have been shown to be achievable in humans (52). Additional preclinical studies designed to address these issues are currently under way.

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References

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