The Multikinase Inhibitor Sorafenib Potentiates TRAIL Lethality in Human Leukemia Cells in Association with Mcl-1 and cFLIP_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 μmol/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+ 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_L, most likely through a translational mechanism, in association with diminished eIF4E phosphorylation, whereas ectopic expression of cFLIP_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_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]
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 DR4 or DR5, 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 sensitizes 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.

Results for the experimental gene were normalized to 18S rRNA levels.

**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% NP-40, 0.5% sodium deoxycholate, 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 antiserum (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 used.
used (CalcuSyn; Biosoft). 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 concentrations ranging from 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., 45–50% 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.

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Detailed time course analysis of sorafenib (7.5 \mu mol/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 ($\Delta \psi_{\text{mito}}$; 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 pro-caspase-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 \mu mol/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 sorafenib and TRAIL revealed similar synergistic interactions (Fig. 1D). Finally, administration of sorafenib and TRAIL in a variety of malignant hematopoietic cell lines, including Raji, Jurkat, and Karpas (human lymphoma); K562 (chronic myelogenous leukemia); and U266 cells (multiple myeloma; Supplementary Fig. S3); or in primary AML specimens (Supplementary Fig. S4B), revealed similar synergistic interactions. Interestingly, neither sorafenib nor TRAIL alone or in combination significantly increased the extent of cell death in normal bone marrow mononuclear or CD34+ cells treated in an identical manner (Supplementary Fig. S4B). Collectively, these findings indicate that sorafenib pretreatment markedly potentiates TRAIL lethality, reflected by induction of mitochondrial injury, caspase activation, and apoptosis.

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. 2B). Furthermore, in sorafenib-pretreated cells, TRAIL exposure induced a pronounced reduction in expression of the BH3 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 of markers of mitochondrial injury and caspase activation (Supplementary Fig. S4).

**Figure 2.** Antiapoptotic and proapoptotic proteins in sorafenib/TRAIL–treated U937 cells. A, U937 cells were exposed to sorafenib (7.5 \mu g), TRAIL (75 ng/mL), or their combination for the indicated time intervals, and at the end cells were pelleted, lysed, and 30 \mu g 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 \mu g 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 pelletted 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.

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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 synergism, given the inability of ectopic Mcl-1 expression to block sorafenib/TRAIL lethality completely, U937 cells expressing a dominant-negative form of procaspase-8 (U/C8DN; ref. 18) were used. As shown in Fig. 4A (left), the pronounced induction of apoptosis by sorafenib/TRAIL was partially but significantly attenuated by enforced expression of dominant-negative procaspase-8 (P < 0.005). As anticipated, activation of procaspase-8 was abolished in U/C8DN cells as was Bid activation and the appearance of tBid (Fig. 4B). Notably, Mcl-1 down-regulation was equivalent in U937/empty vector control cells and U937/C8DN cells, indicating that this phenomenon occurred independently of the extrinsic cascade (Fig. 4C). Interestingly, the ability of the sorafenib/TRAIL regimen to induce Bak and Bax conformational change was substantially attenuated in lysates from U/C8DN cells (Fig. 4C), arguing that in sorafenib-pretreated cells, receptor pathway activation by TRAIL plays a critical functional role in synergism between these agents. Moreover, given the intrinsic TRAIL resistance of U937 cells, they suggest 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 potentiation of TRAIL-induced Bak and Bax conformational change and lethality, and that Bak activation may be a particularly important contributor to synergism.

Interactions between sorafenib and TRAIL require a functional receptor-mediated apoptotic pathway. To determine whether additional mechanisms might be involved in sorafenib/TRAIL synergism, given the inability of ectopic Mcl-1 expression to block sorafenib/TRAIL lethality completely, U937 cells expressing a dominant-negative form of procaspase-8 (U/C8DN; ref. 18) were used. As shown in Fig. 4A (left), the pronounced induction of apoptosis by sorafenib/TRAIL was partially but significantly attenuated by enforced expression of dominant-negative procaspase-8 (P < 0.005). As anticipated, activation of procaspase-8 was abolished in U/C8DN cells as was Bid activation and the appearance of tBid (Fig. 4B). Notably, Mcl-1 down-regulation was equivalent in U937/empty vector control cells and U937/C8DN cells, indicating that this phenomenon occurred independently of the extrinsic cascade (Fig. 4C). Interestingly, the ability of the sorafenib/TRAIL regimen to induce Bak and Bax conformational change was substantially attenuated in lysates from U/C8DN cells (Fig. 4C), arguing that in sorafenib-pretreated cells, receptor pathway activation by TRAIL plays a critical functional role in synergism between these agents. Moreover, given the intrinsic TRAIL resistance of U937 cells, they suggest 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 potentiation of TRAIL-induced Bak and Bax conformational change and lethality, and that Bak activation may be a particularly important contributor to synergism.

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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. The 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
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), suggesting that sorafenib-mediated cFLIPL down-regulation plays a functional role in sensitizing U937 cells to TRAIL.

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 cFLIP L expression (Fig. 5D), suggesting that perturbations in these proteins act independently and cooperatively to promote apoptosis in sorafenib/TRAIL–treated cells.

**Sorafenib-mediated cFLIP L down-regulation involves a translational component.** cFLIP L 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 cFLIP L 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 cFLIP L was only minimally restored. These findings indicate that although a contribution of caspase-mediated degradation to the down-regulation of cFLIP L after TRAIL administration cannot be completely excluded, it is unlikely to represent the major mechanism of reduced FLIP L expression. Moreover, no changes in cFLIP L 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 cFLIP L down-regulation. Finally, levels of cFLIP L mRNA were determined by real-
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 cFLIPL expression primarily at the transcriptional level, combined exposure to actinomycin D and sorafenib would have little further effect on cFLIPL down-regulation; conversely, simultaneous inhibition of both transcription and translation should have a greater effect on cFLIPL levels than blocking transcription alone (i.e., by actinomycin D). As shown, coexposure of cells to sorafenib and actinomycin D reduced cFLIPL 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 cFLIPL 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 cFLIPL, 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 cascade (41). Following activation of procaspase-8 at the level of the DISC, death signals are transmitted to the mitochondria via cleavage of Bid to generate tBid. tBid then interacts with Bax and Bak to promote their oligomerization and insertion into the outer mitochondrial membrane, leading to mitochondrial outer membrane permeabilization (41, 42). Previously, we and others reported that sorafenib lethality in human leukemia cells could be explained in part through Mcl-1 down-regulation (5, 6), and that this phenomenon was associated with the inhibition of Mcl-1 translation (3, 5). In view of these findings, it is likely 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 coadministration 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 cFLIPa (26 kDa), cFLIPb (a 24-kDa form that was isolated from Raji cells), and the 55 kDa cFLIP (review in ref. 46). Both cFLIPa and cFLIPb may form heterodimers with caspase-8, thereby functioning as caspase-8 dominant-negative inhibitors (46). Although no changes were observed in the short form of cFLIP, sorafenib induced a dramatic decrease in cFLIPb that in all likelihood potentiated activation of the extrinsic apoptotic cascade.

Diverse mechanisms have been implicated in the control of cFLIP expression including transcriptional regulation through nuclear factor-κB (34, 35) or, at the protein level, by modulation of both proteasome- and caspase-mediated degradation (30, 37). However, it is important to note that neither proteasome nor caspase inhibition significantly restored cFLIP expression to basal levels in sorafenib/TRAIL–treated cells. Similarly, the modest reduction (i.e., ~25%) in levels of cFLIPb-specific mRNA in sorafenib/TRAIL–treated cells was unlikely to account for the early and virtually complete down-regulation of cFLIPb observed at the protein level. Collectively, these observations suggest that factors other than or in addition to inhibition of transcription contribute to abrogation of cFLIPb expression in sorafenib/TRAIL–treated cells, and that posttranslational (ubiquitination, proteasome degradation) or caspase-dependent mechanisms are at most only partially involved. They also suggest that the simultaneous down-regulation of Mcl-1 and FLIPb by sorafenib may provide a unique mechanism by which otherwise TRAIL-resistant leukemia cells may be sensitized to this agent.

The present results suggest that sorafenib-mediated inhibition of translation, possibly stemming from inactivation of the initiation factor eIF4E, represents an important component of cFLIPb down-regulation, analogous to sorafenib-induced Mcl-1 down-regulation in human leukemia cells (5). Sorafenib-mediated Mcl-1 down-regulation and inhibition of eIF4E phosphorylation have also recently been observed in human hepatocellular carcinoma cells (3). Notably, in the present study, inhibition of eIF4E phosphorylation was observed in cells exposed to a relatively low, marginally toxic concentration of sorafenib. eIF4E represents the rate-limiting component of cap-dependent translation initiation, binds the methyl-7-guanosine cap at the 5′ untranslated region of processed mRNA, and transports transcript to the ribosome (47). Its activity, which is tightly regulated, plays an important role in cell growth and transformation, as eIF4E dysregulation promotes inappropriate translation of mRNA (48). Moreover, eIF4E has been implicated in cancer pathogenesis (49). Collectively, these findings suggest that eIF4E may represent a valid target for therapeutic intervention. Given that both Mcl-1 and cFLIPb are short-lived proteins, it follows that their expression might be particularly susceptible to sorafenib-mediated translation inhibition. However, because sorafenib-mediated translation inhibition is most likely a global phenomenon, the possibility that interference with the synthesis of other proteins contributes to sorafenib/TRAIL lethality cannot be excluded. The findings that in sorafenib-treated cells, cFLIPb expression was substantially reduced at early intervals (e.g., 4–6 h) during which mRNA levels were unperturbed under conditions of caspase inactivation, and that sorafenib significantly reduced cFLIPb expression in actinomycin-treated cells, suggest that as in the case of Mcl-1, inhibition of transcription plays either no or a relatively minor role in sorafenib-mediated cFLIPb down-regulation. Lastly, very recent studies from our group have uncovered a novel mechanism of sorafenib-mediated lethality which involves the induction of ER stress in association with phosphorylation/inactivation of the translation initiation factor eIF2α (50). 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 down-regulate the key antiapoptotic proteins Mcl-1 and cFLIP<sub>L</sub>, 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<sub>L</sub>. By disrupting the translational machinery and reducing expression 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<sub>L</sub>, 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,

References


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