MCL-1 as a Buffer for Proapoptotic BCL-2 Family Members during TRAIL-induced Apoptosis

A MECHANISTIC BASIS FOR SORAFENIB (BAY 43-9006)-INDUCED TRAIL SENSITIZATION*‡§

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Previous studies have suggested that Mcl-1, an antiapoptotic Bcl-2 homolog that does not exhibit appreciable affinity for the caspase 8-generated C-terminal Bid fragment (tBid), diminishes sensitivity to tumor necrosis factor-α-related apoptosis-inducing ligand (TRAIL). This study was performed to determine the mechanism by which Mcl-1 confers TRAIL resistance and to evaluate methods for overcoming this resistance. Affinity purification/immunoblotting assays using K562 human leukemia cells, which contain Mcl-1 and Bcl-xL, as the predominant antiapoptotic Bcl-2 homologs, demonstrated that TRAIL treatment resulted in binding of tBid to Bcl-xL but not Mcl-1. In contrast, TRAIL caused increased binding between Mcl-1 and Bak that was diminished by treatment with the caspase 8 inhibitor N-(N-acetyl(isoleucyl glutamylthreonyl) aspartic acid (O-methyl ester)-fluoromethyl ketone (IETD(OMe)-fmk) or the c-Jun N-terminal kinase inhibitor SP600125. In addition, TRAIL caused increased binding of Bim and Puma to Mcl-1 that was inhibited by IETD(OMe)-fmk but not SP600125. Further experiments demonstrated that down-regulation of Mcl-1 by short hairpin RNA or the kinase inhibitor sorafenib increased TRAIL-induced Bak activation and death ligand-induced apoptosis in a wide variety of neoplastic cell lines as well as clinical acute myelogenous leukemia specimens. Collectively, these observations not only suggest a model in which Mcl-1 confers TRAIL resistance by serving as a buffer for Bak, Bim, and Puma, but also identify sorafenib as a potential modulator of TRAIL sensitivity.

The cytotoxic cytokine TRAIL, as well as agonistic antibodies against its two receptors, death receptor (DR) 4 and DR5, are currently undergoing extensive preclinical and early clinical testing in patients with hematological malignancies and solid tumors (1–4). Interest in these agents stems from the observation that TRAIL kills a wide variety of neoplastic human cells with limited toxicity in normal cells in vitro and in vivo (5–7). This selectivity appears to reflect the physiological function of TRAIL as a participant in immune system-mediated destruction of neoplastic cells (1, 8). Consistent with this postulated function, TRAIL is expressed on interferon-activated natural killer cells as well as monocytes, T cells, and dendritic cells (1, 8).

TRAIL and agonistic anti-TRAIL receptor antibodies kill target cells by triggering the extrinsic caspase activation pathway. According to current understanding, the binding of TRAIL or agonistic antibodies to the extracellular domains of DR4 and/or DR5 leads to recruitment and oligomerization of the adaptor molecule FADD at the receptor cytoplasmic domains (11–14). Oligomerized FADD in turn binds and activates procaspases 8 and 10. Once activated, these initiator caspases cleave a small number of substrates, including the proapoptotic Bcl-2 family member Bid and the zymogen form of the major effector caspase, caspase 3 (15). In so-called type I cells, the amounts of caspase 8 and caspase 3 that are activated downstream of FADD are sufficient to trigger the entire apoptotic process (16). In type II cells, on the other hand, the amount of caspase 8 that is initially activated is insufficient to trigger full-blown apoptosis (16). Instead, caspase 8-mediated cleavage of Bid ostensibly leads to mitochondrial release of cytochrome c, which participates in amplification of the death signal through caspase 9 activation (11–13). Concomitant release of mitochondrial XIAP antagonists in type II cells is thought to facilitate apoptosis by relieving XIAP-mediated caspase inhibition (17–19). Conversely, overexpression of antiapoptotic Bcl-2 family mem-
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bers such as Bcl-2 or Bcl-xL, which bind tBid and inhibit mitochondrial permeabilization, has been observed to inhibit TRAIL-induced apoptosis in type II cells (20–25).

Recent studies have also implicated Mcl-1 in the regulation of TRAIL-induced apoptosis (26–29). In particular, siRNA- or shRNA-mediated Mcl-1 down-regulation enhances TRAIL cytotoxicity in cholangiocarcinoma or hepatocellular carcinoma cells (27–29). Several explanations for this phenomenon have been proposed. On the one hand, it has been suggested that Mcl-1 down-regulation frees the proapoptotic Bcl-2 family member Bim, which then binds to Bax to induce apoptosis (29). This explanation, however, is difficult to reconcile with earlier studies that failed to implicate Bim as a prominent factor involved in death ligand-induced mitochondrial permeabilization (30, 31). Alternatively, it has also been reported that Mcl-1 binds directly to tBid, inhibiting its ability to facilitate mitochondrial permeabilization (32). This model, however, conflicts with affinity measurements that demonstrate a >10 μM \( K_d \) value for the interaction between Mcl-1 and the Bid BH3 domain (33, 34). Accordingly, the mechanistic basis for the ability of Mcl-1 to modulate TRAIL-induced apoptosis requires further investigation.

Additional studies have demonstrated that JNK, a kinase that modulates the mitochondrial apoptotic pathway in other contexts (35–37), is activated in a caspase 8-dependent manner during TRAIL exposure (38 – 46). In several model systems this TRAIL-induced JNK activation contributes to TRAIL cytotoxicity (40, 45, 46), most likely by activating (47) the proapoptotic Bcl-2 family member Bim (46). In other model systems, on the other hand, a contribution of JNK activation to the cytotoxicity of TRAIL has been difficult to demonstrate (42, 43), suggesting that its mechanism and role might be context-dependent.

To gain further insight into the ability of Mcl-1 to modulate death receptor-induced apoptosis, we have examined changes in the binding of proapoptotic Bcl-2 family members to Mcl-1 during the course of TRAIL-induced apoptosis. Results of this analysis demonstrated increased binding of Bak, Bim, and Puma after TRAIL treatment, we further examined the effect of Mcl-1 down-regulation (49, 50), enhances the cytotoxic effects of TRAIL and other death receptor ligands in a variety of neo-plastic human cell lines and in primary AML specimens.

EXPERIMENTAL PROCEDURES

Materials—Sorafenib was a kind gift from Chris Carter, Bayer Corp. (West Haven, CT). Additional reagents were purchased from the following suppliers: recombinant human TRAIL and agonistic anti-DR4 antibody 71903 (MAB631) from R & D Systems (Minneapolis, MN); CH-11 agonistic anti-Fas antibody from Upstate Biotechnology, Inc. (Lake Placid, NY); the broad spectrum caspase inhibitor Z-VAD(OMe)-fmk (51) from Biomol (Plymouth Meeting, MA); the caspase 8 inhibitor IETD(OMe)-fmk from Enzyme Systems Products (Dublin, CA); the JNK inhibitor SP600125 from EMD Biosciences (San Diego); BMH from Pierce; and allophycocyanin (APC)-conjugated annexin V from Pharmingen.

Antibodies for immunoblotting or flow cytometry were obtained from the following suppliers: murine monoclonal antibodies that recognize XIAP, Mcl-1, Bcl-xL, caspase 3, and caspase 8 from BD Biosciences; monoclonal anti-Fas (apo-1-1), anti-DR4 (HS101), and anti-DR5 (HS201) from Alexis (San Diego, CA); murine Ab-1 monoclonal antibody to active Bak from Calbiochem; murine monoclonal anti-Bcl-2 and rabbit polyclonal anti-Mcl-1 from Dako (Carpinteria, CA); rabbit anti-Bax and goat anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA); rabbit anti-Puma from Azxora (San Diego, CA); rabbit anti-Bak from Upstate Biotechnology, Inc. (Lake Placid, NY); rabbit polyclonal antibodies that recognize Akt, Bid, Bin, Bcl-xL, JNK, and phospho-Thr\(^{183}\)Tyr\(^{185}\) JNK from Cell Signaling Technology (Beverly, MA); and murine anti-FLAG epitope antibody from Sigma. Murine monoclonal antibodies that recognize PARP, heat shock protein 90, and histone H1 were gifts from Dr. G. Poirier (Laval University, Ste-Foy, Quebec, Canada), David Toft (Mayo Clinic, Rochester, MN), and James Sorace (Veterans Affairs Medical Center, Baltimore), respectively.

Cell Culture—All cell lines were maintained in medium containing 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine. K562 cells were maintained in medium A, which consisted of RPMI 1640 medium with 5% (v/v) FBS,Jurkat cells (from Paul Leibson, Mayo Clinic, Rochester, MN), HL-60 cells, and T98G cells (American Type Culture Collection) were maintained in RPMI 1640 medium containing 10% (v/v) FBS (medium B). K562 cells (from Joya Chandra, M. D. Anderson Cancer Center, Houston, TX) were maintained in Iscove’s modified Dulbecco’s medium containing 10% (v/v) FBS (medium C).

Plasmid Construction and Site-directed Mutagenesis— pcDNA3.1 containing the full-length Mcl-1 cDNA was a kind gift from Dr. Ruth Craig (Dartmouth Medical School, Hanover, NH). To generate plasmid encoding tagged Mcl-1 for S protein-agarose pulldowns, a 15-amino acid S peptide tag (KETAAK - FERQHMD) with GA linker (total 28 amino acids) was inserted at the N terminus of wild-type Mcl-1 essentially as described (52). To allow re-expression of untagged Mcl-1 in cells expressing Mcl-1 shRNA, the wild-type Mcl-1 cDNA was rendered shRNA-resistant by mutating the cDNA to CGC- GATTGCTGTCAAAGC to induce silent mutations (indicated by underlines) of the third nucleotides in five of the targeted codons using a QuickChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) following the supplier’s instructions. A plasmid encoding S peptide-tagged Bcl-xL was constructed by isolating cDNA encoding codons 2–234 of the Bcl-xL open reading frame and inserting the cDNA into the Ascl and EcoRI sites of pSP6 (52). Full-length Bid cDNA, amplified by reverse transcription-PCR of total RNA extracted from K562 cells, was cloned in-frame with FLAG into the pc12 vector using EcoRI and NotI sites. Bak shRNA targeted the sequence 5’-GTACGAGATTTTCAAT-3’ using the vector pCMS4A-EGFP. All plasmids were subjected to automated sequencing to verify the described alteration and confirm that no additional mutations were present. These plasmids were
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Prepared for transfection using a plasmid maxiprep kit (Qiagen, Valencia, CA).

Transfections—For transient transfections with shRNA, 1 × 10^7 K562 cells were transfected with 40 μg of empty pSSH1P vector or vector encoding a previously described Mcl-1 shRNA (27) (a kind gift from Greg Gores) and 5 μg of plasmid encoding enhanced green fluorescent protein (EGFP) fused to histone H2B (a kind gift from Jan van Deursen, Mayo Clinic, Rochester, MN) using a BTX 820 square wave electroporator (BTX, San Diego, CA) programmed to deliver a 10-ms pulse at 320 V. Under these conditions K562 cells have a 60–80% transfection efficiency when monitored by flow cytometry for EGFP fluorescence. In some experiments, 20 μg of pcDNA3.1 without an insert or with cDNA encoding siRNA-resistant Mcl-1 was included as indicated. 24 h after transfection, cells were sorted based on EGFP expression and prepared for SDS-PAGE as described previously (53). Alternatively, 24 h after transfection, cells were treated for 24 h with the indicated concentrations of TRAIL. At the completion of the incubation, cell death was assessed using APC-coupled annexin V as described below. Data were analyzed by gating on cells that were EGFP-positive.

Transient transfections with plasmid encoding FLAG epitope-tagged Bid were performed in a similar manner using 40 μg of plasmid DNA. 24 h after transfection, cells were utilized for pulldown assays as described below.

To generate stable cell lines, aliquots containing 1 × 10^7 K562 cells were transfected with 40 μg of plasmid encoding S peptide-tagged Mcl-1 or Bcl-xL, as described above. 48 h after transfection, cells were transferred to medium A containing 800 μg/ml G418. After the G418-resistant cells grew to 5 × 10^6/ml, clones were isolated by limiting dilution and assayed for transgene expression by blotting with anti-S peptide monoclonal antibody (52).

Pulldown Assays—To assess changes in binding of partners to Mcl-1 and Bcl-xL, K562 cells expressing S peptide-tagged Mcl-1 or Bcl-xL were treated with 25 ng/ml TRAIL for 0–5 h. At the completion of the incubation, cells were solubilized at 4 °C for 30 min in lysis buffer consisting of 1% (w/v) CHAPS, 20 mM HEPES (pH 7.4), 150 mM NaCl, 1% (v/v) glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 100 μM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, and 20 mM microcystin. After centrifugation at 14,000 × g for 15 min to sediment insoluble material, aliquots of supernatant containing 1000 μg of protein were incubated overnight at 4 °C with 40 μl of S protein-agarose (EMD Biosciences, San Diego) and washed five times in lysis buffer. Polypeptides bound to the beads were released by heating for 20 min at 65 °C in SDS sample buffer consisting of 4 M urea, 2% (w/v) SDS, 62.5 mM Tris-HCL (pH 6.8), 1 mM EDTA, and 5% (v/v) 2-mercaptoethanol. After blots were probed with antibodies (53), films from at least three independent experiments were scanned on a Hewlett Packard Scanjet 4C scanner and quantified using ImageJ software. After normalization for Mcl-1 content, results were analyzed using paired t tests and presented as the mean ± S.D. of the indicated number of experiments.

Assays of Apoptosis—After treatment with the indicated concentrations of JNK inhibitors, sorafenib and/or TRAIL, cells were washed, lysed at 4 °C in buffer consisting of 0.1% (w/v) Triton X-100 and 50 μg/ml propidium iodide in 0.1% (w/v) sodium citrate, and subjected to flow cytometry as described previously to assess DNA fragmentation (54, 55). In parallel experiments, cells were fixed in 3:1 (v/v) methanol/acetic acid, stained with 1 μg/ml Hoechst 33258, and examined by fluorescence microscopy for apoptotic changes in nuclear morphology (56). In further experiments, cells were stained with APC-conjugated annexin V and 0.1 μg/ml propidium iodide in 140 mM NaCl, 2.5 mM CaCl_2, and 10 mM HEPES (pH 7.4) as instructed by the supplier. For this assay, 30,000 events were collected from the FL2 (excitation 488 nm, emission 585 ± 21 nm) and FL4 (excitation 635 nm, emission 661 ± 8 nm) channels of a BD Biosciences FACSCalibur flow cytometer and analyzed using Cellquest software (BD Biosciences).

Clonogenic Assays—Aliquots containing 0.25 × 10^6 K562 cells in 1 ml of medium A were incubated with the indicated concentrations of sorafenib and TRAIL for 24 h. Cells were then sedimented at 80 × g for 5 min, resuspended in fresh medium A, diluted, and plated in 35-mm grid plates in the medium of Pike and Robinson (57) containing 0.3% (w/v) Bacto agar (BD Biosciences). After incubation for 10–14 days at 37 °C, colonies containing ≥50 cells were counted on an inverted microscope.

Alternatively, aliquots containing 250 T98G cells were plated in replicate 35-mm tissue culture plates and allowed to adhere for 14 h. After treatment with the indicated concentration of sorafenib and agonistic anti-DR5 antibody for 24 h, cells were washed, incubated for 7–8 days, stained with Coomassie Blue, and manually examined for colony formation. Diluent-treated plates typically contained 150 colonies.

Analysis of Cell Surface Death Receptor Expression—1 × 10^6 cells were stained with mouse apo-1-1 anti-Fas, HS101 anti-DR4, or HS201 anti-DR5 on ice for 45 min. After two washes in PBS containing 2% (v/v) FBS, cells were incubated with phycoerythrin-conjugated anti-mouse IgG for an additional 30 min on ice. Following washing, cells were fixed in 1% paraformaldehyde and stored in the dark at 4 °C until analyzed by flow cytometry.

Analysis of Active Bak by Flow Cytometry and Cross-linking—Aliquots containing 1 × 10^6 cells were washed twice with PBS and fixed with 1% (w/v) parafomaldehyde for 5 min at 20 °C. After a wash with PBS containing 2% (v/v) FBS, cells were resuspended in 100 μl of 0.15% saponin in PBS containing mouse monoclonal Ab-1 anti-Bak and incubated for 30 min on ice. After two washes with PBS containing 2% (v/v) FBS, cells were incubated with phycocerythrin-conjugated mouse IgG for 30 min on ice and examined by flow microfluorimetry. Mean fluorescence intensities of labeled cells were calculated using Cellquest software (BD Biosciences).

To provide an alternative assessment of Bak activation, mitochondria were isolated from Jurkat cells and cross-linked with BMH. In brief, after treatment cells were washed in PBS; resuspended in ice-cold Buffer H consisting of 25 mM HEPES (pH 7.4 at 4 °C), 5 mM MgCl_2, 1 mM EGTA, 1 mM EDTA, 1 mM sodium vanadate, 20 mM microcystin, 1% (w/v) thiodiglycol, 1 mM α-phenylmethylsulfonyl fluoride, 100 units/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin A; incubated on ice for 20 min; and lysed with 30 strokes in a tight-fitting Dounce homogenizer. After lysates were clarified at 2300 × g for 5 min,
m isolation of Bak and Bax as the principal effectors of mitochondrial permeabilization. For these experiments, we stably transfected SK62 cells with an S-peptide-tagged version of Mcl-1 so that complexes could be isolated without the use of antibodies (52), thereby diminishing interference of immunoglobulin light chain with subsequent detection of proapoptotic Bcl-2 family members, which are similar in size. The initial experiments also focused on the first 3–5 h after TRAIL addition to study events that occurred before the cells became frankly apoptotic.

As indicated in Fig. 1A, the clone used for these pulldown experiments contained roughly equal levels of endogenous and S-peptide-tagged Mcl-1. When these cells were treated with TRAIL, increased Bak was detected in the Mcl-1 pulldowns within 1–3 h (Fig. 1B, left panel), with a 2.2 ± 0.5-fold increase (n = 6, p = 0.002) in the signal for Bak bound to Mcl-1 at 3 h (Fig. 1B, right panel). In contrast, Bak was not detected in these Mcl-1 pulldowns even though it was readily detected in whole cell lysates (Fig. 1B, right panel). Further experiments demonstrated that after TRAIL treatment—based on previous experiments suggesting that Mcl-1 affects TRAIL sensitivity, we examined TRAIL-induced changes in binding of Bcl-2 family members to Mcl-1. These experiments focused on SK62 cells, which were derived from a patient in the blast crisis phase of chronic myelogenous leukemia, because this cell line is relatively resistant to TRAIL (see below). These cells also express high levels of Bcl-xL, but relatively low levels of Bcl-2 (63, 64) and A1, simplifying subsequent analysis of Bcl-2 family member interactions.

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the binding of Bak to S peptide-tagged Mcl-1 was abolished when Arg<sup>263</sup>, a conserved residue in the BH3 binding pocket of Mcl-1, was mutated to alanine (Fig. 1C), ruling out the possibility that the interaction detected in these pulldown assays reflects the nonspecific aggregation of hydrophobic domains upon extraction of Bak and Mcl-1 from biological membranes.<sup>5</sup> IETD(OMe)-fmk, which exhibits selectivity for caspases 8 and 10 (65), prevented the increased binding of Bak to Mcl-1 (Fig. 1, D and E), suggesting that this is an early event downstream of caspase 8 activation.

In addition to caspase activation, TRAF2-mediated initiation of stress-activated kinase signaling (40–43) is observed after TRAIL-induced DISC formation. In K562 cells, as in other cell types, JNK isoforms were phosphorylated during the first several hours after TRAIL exposure (Fig. 2A). To assess the potential importance of this TRAIL-induced JNK activation in Bak trafficking and subsequent apoptosis, cells were treated with TRAIL in the absence or presence of the JNK inhibitor SP600125 (66). As indicated in Fig. 2, B and C, SP600125 prevented the TRAIL-induced binding of Bak to Mcl-1. Moreover, when Bak did not bind to Mcl-1, increased TRAIL-induced Bak activation was observed (Fig. 2, D and E), as evidenced by the 2.3 ± 0.4-fold increase in mean fluorescence intensity when permeabilized cells were stained with antibody that recognizes the active conformation of Bak (n = 3; p = 0.01). Concomitant with this change, enhanced TRAIL-induced apoptosis (Fig. 2, F and G) was observed in the presence of SP600125. In particular, the percentage of cells with “subdiploid” DNA content after treatment with 50 ng/ml TRAIL increased from 18 ± 10% in the absence of SP600125 to 45 ± 7% in the presence of SP600125 (n = 4; p = 0.0005).

These effects of SP600125 were not limited to K562 cells. Instead, SP600125 also increased TRAIL-induced apoptosis in HL-60 acute myelogenous leukemia cells (Fig. 3A) and Jurkat acute lymphocytic leukemia cells (Fig. 3B). For example, 25 ng/ml TRAIL induced apoptosis in 17 ± 7% of HL-60 cells in the absence of SP600125 and 34 ± 6% in the presence of SP600125 (n = 3; p = 0.04). Likewise, 12.5 ng/ml TRAIL induced apoptosis in 33 ± 10% of Jurkat cells in the absence of SP600125 and 54 ± 8% in the presence of SP600125 (n = 3; p = 0.03). Similar effects were observed with N-(4-amino-5-cyano-6-ethoxyprpyridin-2-yl)-2-(2,5-dimethoxy-phenyl) acetamide, a structurally unrelated JNK inhibitor (67).

As was the case in K562 cells, the increased apoptosis in Jurkat cells paralleled increased Bak activation, as evidenced by the 1.9 ± 0.4-fold increase (n = 3, p = 0.01) in mean fluorescence intensity when permeabilized cells were stained with antibody that recognizes the active conformation of Bak (Fig. 3, C and D). To confirm that this increased binding of anti-active Bak antibody truly reflected an alteration in Bak activation, we also examined Bak oligomerization by isolating mitochondria and reacting them with the cross-linking agent BMH before blotting with anti-Bak antibodies. Because the original description of this method (68) specified a final BMH concentration (10 mM) that is beyond the limit of solubility in aqueous buffers, we first examined the effects of various BMH concentrations on Bak cross-linking. Consistent with previous results (69), these experiments demonstrated that increasing the cross-linking agent beyond an optimal concentration (Fig. 3E, lanes 2 and 3) leads to decreased cross-linking (Fig. 3E, lanes 4–6), presumably because of derivatization of binding partners by two different BMH molecules rather than cross-linking by a single one. With an optimal concentration, we observed increased Bak oligomers in mitochondria from cells treated with TRAIL + SP600125 compared with TRAIL or SP600125 alone (Fig. 3F, lanes 2–4).

Collectively, the results in Figs. 2 and 3 suggest that TRAIL treatment is associated with increased binding of Bak to Mcl-1 and that Bak activation is enhanced when this binding is diminished by SP600125.

**TRAIL-induced Binding of Bim and Puma but Not tBid to Mcl-1—** Further experiments utilized K562 cells stably expressing S peptide-tagged Mcl-1 (Fig. 1A) to examine the binding of BH3-only polypeptides to Mcl-1. Bim and Puma were studied based on preliminary mass spectrometry results showing constitutive binding of these polypeptides to Mcl-1.<sup>6</sup> tBid was examined based on its involvement in death ligand-induced apoptosis.

As indicated in Fig. 4A (left and right panels), TRAIL treatment resulted in increased recovery of Puma, Bim<sub>11</sub>, and Bim<sub>1</sub> in Mcl-1 pulldowns even though total cellular Puma and Bim levels remained unchanged (Fig. 4A, middle panel). Additional experiments demonstrated that the trafficking of Puma and Bim to Mcl-1 was inhibited by IETD-fmk but not by SP600125 (data not shown).

In contrast to Puma and Bim, tBid was not detected in the Mcl-1 pulldowns (Fig. 4A, lanes 1–4) even though full-length Bid was clearly diminishing (Fig. 4A, lanes 7 and 8). Further analysis demonstrated that a variety of commercially available anti-Bid antibodies, including those reported to detect cleaved Bid, lacked the sensitivity to detect tBid in Jurkat or K562 cells even under conditions where most or all of the full-length Bid has been cleaved (Fig. 4B and data not shown). To circumvent this problem, the fate of a Bid construct containing the FLAG epitope at its C terminus was assessed. As indicated in Fig. 4C, this construct was cleaved to a tagged 15-kDa product corresponding to tBid in a caspase 8-dependent manner during treatment of K562 cells with TRAIL. Even though this cleavage product could be readily detected in whole cell lysates (Fig. 4D, lanes 2 and 3), it was not detected in Mcl-1 pulldowns from the same experiments (Fig. 4D, lanes 5 and 6). In contrast, when the same experiment was performed using K562 cells stably expressing S peptide-tagged Bcl-x<sub>L</sub> (Fig. 4E), FLAG-tagged tBid was readily detectable in Bcl-x<sub>L</sub> pulldowns (Fig. 4F).

**Mcl-1 Levels Affect TRAIL Sensitivity—** Collectively, the results in Figs. 1–4 indicate that a number of proapoptotic Bcl-2 family members, including Bak, Bim, and Puma (but

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<sup>5</sup> Additional experiments also indicated that shRNA-resistant R263A Mcl-1 is significantly less effective than shRNA-resistant wild-type Mcl-1 at protecting cells from Mcl-1 knockdown (data not shown).

<sup>6</sup> S.-H. Lee and S. H. Kaufmann, unpublished observations.

<sup>7</sup> The small amount of tBid bound to Bcl-x<sub>L</sub> in the absence of TRAIL appears to reflect induction of apoptosis by the transfection procedure itself and the resulting activation of caspase 8 downstream of effector caspases (96).
not Bax or tBid), bind to Mcl-1 in increased amounts during TRAIL exposure. If this binding were important in modulating TRAIL sensitivity, one would predict that Mcl-1 down-regulation would, like SP600125 treatment, enhance TRAIL sensitivity. To test this prediction, K562 cells were transfected with empty vector or plasmid encoding Mcl-1 shRNA. Because free EGFP can be lost as cells undergo apoptosis, cells were co-transfected with a plasmid encoding EGFP fused to histone H2B to permit identification and analysis of transfected cells.

FIGURE 2. Role of TRAIL-induced JNK activation in binding of Bak to Mcl-1 and subsequent apoptosis. A, after K562 cells were treated with 25 ng/ml TRAIL for indicated times, whole cell lysates were probed with the indicated antibodies. B, after K562 cells stably expressing S peptide-Mcl-1 were treated with 25 ng/ml TRAIL in the absence or presence of 20 μM SP600125 for the indicated times, Mcl-1 pulldowns were probed with antibodies that recognize Bak or, as a loading control, S peptide-tagged Mcl-1. C, densitometric analysis of four (SP600125) or six (no SP600125) independent experiments depicted in B. **, p = 0.002 by paired t test. D, after treatment for 24 h with 0.1% (v/v) Me2SO (DMSO) (left, gray), 20 μM SP600125 (left, dark line), 0.1% (v/v) Me2SO + 25 ng/ml TRAIL (right, gray), or 20 μM SP600125 + 25 ng/ml TRAIL (right, dark), cells were stained with antibody that recognizes active Bak (97) and analyzed by flow cytometry. E, from data similar to D, mean fluorescence intensities were calculated using CellQuest software and normalized to diluent-treated cells. **, p = 0.01, n = 3. F, after treatment for 24 h with diluent, 50 ng/ml TRAIL, 20 μM SP600125, or 50 ng/ml TRAIL + 20 μM SP600125, K562 cells were stained with PI and analyzed by flow microfluorimetry. Note the increase in particles with >2n DNA content with the combination. Both samples treated with SP600125 exhibit a G2 arrest as described previously (98). G, K562 cells were treated with the indicated concentrations of TRAIL in the absence (open circles) or presence (closed circles) of 20 μM SP600125 for 24 h, stained with PI, and analyzed for subdiploid events as illustrated in F.
Beginning 24 h after transfection, cells were either sorted based on EGFP-histone H2B content and subjected to immunoblotting or treated with TRAIL and analyzed for apoptosis by flow cytometry, again gating on the EGFP-histone H2B-expressing cells. Immunoblotting demonstrated that Mcl-1 shRNA induced selective down-regulation of Mcl-1 without affecting Bcl-xL, Bax, Bak, Bid, Puma, or Bim (Fig. 5A). Bcl-2, which is expressed at low levels in K562 cells (63, 64), was below the level of detection in this experiment. This selective down-regulation of Mcl-1 was accompanied by marked sensitization to TRAIL (Fig. 5B). In particular, in experiments where Mcl-1 shRNA by itself had little effect on cell survival, treatment with 25 ng/ml TRAIL induced apoptosis in 53 ± 4% (mean ± S.D., n = 5) of cells transfected with Mcl-1 shRNA compared with 15 ± 13% of cells transfected with empty vector (p = 0.004). Subsequent experiments demonstrated that co-transfection with shRNA-resistant Mcl-1 (Fig. 5C, inset) resulted in diminished TRAIL sensitivity, establishing that the effect of the Mcl-1 shRNA was because of altered Mcl-1 content rather than an off-target effect of the hairpin RNA (Fig. 5C). Moreover, Bak shRNA (Fig. 5D) diminished the ability of Mcl-1 shRNA to enhance TRAIL sensitivity (Fig. 5E), establishing that Bak plays a critical role in the ability of Mcl-1 knockdown to enhance TRAIL sensitivity.

Sorafenib Sensitizes Jurkat Cells to TRAIL, Anti-DR5, and Agonistic Anti-Fas Antibodies—The results in Fig. 5 suggest that Mcl-1 down-regulation might be an effective means of sensitizing cells to TRAIL. In further experiments, a pharmacological approach was utilized to extend this observation to additional cell lines and additional death ligands. These studies employed sorafenib, a recently approved kinase inhibitor that down-regulates Mcl-1 (49, 50), most likely by inhibiting the translation of its message (50).

Jurkat cells, which are sensitive to a number of death ligands, were initially used for these experiments. As illustrated in Fig. 6A, treatment with 5 μM sorafenib, a concentration that is readily achieved in the clinical setting, increased the amount of TRAIL-induced apoptosis. Because sorafenib by itself induced some toxicity, the effects of the TRAIL/sorafenib combination were analyzed by the median effect method (61). This analysis demonstrated synergy between these agents, as indicated by a combination index below 1.0 at most concentrations (Fig. 6B). Once again this increased apoptosis reflected increased Bak activation as detected using the conformation-sensitive Bak antibodies.
Bcl-2 Family Trafficking during TRAIL-induced Apoptosis

Effects of the Sorafenib on TRAIL Receptor-mediated Apoptosis in Other Cell Lines—To rule out the possibility that these results were unique to Jurkat cells, we next examined the effect of sorafenib and TRAIL on K562 cells, which are 30-fold less sensitive to this death ligand (cf. Fig. 6A and Fig. 8). Even though TRAIL and sorafenib individually induced DNA fragmentation in fewer than 20% of K562 cells, the combination induced DNA fragmentation in 80% of cells (Fig. 8A and B). Analysis by the median effect method demonstrated that the effects of the two agents were highly synergistic, with combination index values of 0.3 over a broad range of cytotoxicity in three separate experiments. Because DNA fragmentation can also be observed when cells undergo necrosis, cells were also stained with Hoechst 33342 and examined for apoptotic morphological changes. As indicated in Fig. 8, A and B, sorafenib induced little apoptosis by itself but nonetheless markedly enhanced the TRAIL-induced apoptosis. Once again, synergy was observed (Fig. 8E), with a combination index of 0.24 ± 0.11 at the LD_{50} and 0.08 ± 0.06 at the LD_{90} values (mean ± S.D., n = 3). Similar
FIGURE 5. Effect of Mcl-1 shRNA on TRAIL-induced apoptosis. A, 24 h after transfection with empty vector and EGFP-histone H2B (lane 1) or Mcl-1 shRNA and EGFP-histone H2B (lane 2), K562 cells were sorted based on EGFP fluorescence. Whole cell lysates prepared from 200,000 sorted cells were probed with antibodies to the indicated polypeptides. Akt served as a loading control. B, 24 h after transfection with empty vector and EGFP-histone H2B (open circles) or Mcl-1 shRNA and EGFP-histone H2B (closed circles), K562 cells were treated for 24 h with the indicated concentrations of TRAIL and stained with allophycocyanin-conjugated annexin V. The percentage of EGFP-positive cells that stained with annexin V is indicated. C, 24 h after transfection with a total of 65 μg of plasmid DNA consisting of 5 μg encoding EGFP-histone H2B in each sample along with 40 μg of empty pSSH1P and 20 μg of empty pcDNA3.1 (open circles), 40 μg of pSSH1P encoding Mcl-1 shRNA and 20 μg of empty pcDNA3.1 (closed circles), or 40 μg pSSH1P encoding Mcl-1 shRNA and 20 μg of pcDNA3.1 containing shRNA-resistant Mcl-1 cDNA (triangles), K562 cells were sorted based on EGFP fluorescence and subjected to immunoblotting (inset, C) or treated for 24 h with the indicated concentrations of TRAIL and stained with annexin V. The percentage of EGFP-positive cells that stained with annexin V is indicated. D, K562 cells transiently transfected with empty vector encoding EGFP-histone H2B (lane 1) or the same plasmid encoding Bak shRNA (lane 2) were incubated for 24 h and sorted for EGFP-positive cells. Whole cell lysates were subjected to SDS-PAGE followed by immunoblotting for the indicated polypeptides. E, K562 cells were transiently transfected with plasmid encoding EGFP-histone H2B and empty vector (1st column) or plasmid encoding Mcl-1 shRNA (2nd and 4th columns) and/or plasmid encoding Bak shRNA (3rd and 4th columns). 24 h after transfection, cells were treated for 24 h with 50 ng/ml TRAIL, then stained with allophycocyanin-coupled annexin V, and examined by two-color flow cytometry. Shown is the percentage of EGFP-Histone H2B0-positive cells that also bind annexin V.

results were obtained when phosphatidylserine externalization, another marker of apoptosis, was assessed using annexin V.4 When cells treated with the individual agents or the combination were blotted for caspases and caspase substrates, the amounts of procaspases 8, 9, and 3 cleaved in response to the combination were much larger than the amounts cleaved after amounts of procaspases 8, 9, and 3 cleaved in response to the other agent alone (Fig. 8F, lane 4). Consistent with these results, the caspase substrate poly(ADP-ribose) polymerase also underwent substantially more cleavage in cells treated with the combination than with either drug alone (Fig. 8F).

To evaluate further the possibility that sorafenib might be affecting TRAIL sensitivity by a mechanism other than Mcl-1 down-regulation, the samples shown in Fig. 8F were blotted for Bcl-2 family members and other apoptotic regulators. This analysis demonstrated sorafenib-induced down-regulation of Mcl-1 as reported previously but no change in Bcl-xL, Bax, or Bak (supplemental Fig. S1). Mcl-1 down-regulation also occurred in the presence of the broad spectrum caspase inhibitor Z-VAD(OMe)-fmk (supplemental Fig. S1, lane 5), ruling out the possibility that Mcl-1 down-regulation merely reflected caspase-mediated cleavage. In contrast, down-regulation of XIAP, a caspase inhibitor also implicated in TRAIL resistance (7, 72–77), was abrogated by Z-VAD(OMe)-fmk (cf. Fig. S1, lanes 4 and 5), suggesting that XIAP down-regulation is caspase-mediated. Levels of c-FLIP, another molecule implicated in TRAIL resistance (22, 24, 78–81), failed to change in K562 cells after sorafenib treatment. Further experiments demonstrated that recruitment of FADD and procaspase 8 to the TRAIL DISC was unaltered in the presence of sorafenib.8

To rule out the possibility that sorafenib was merely accelerating the kinetics of TRAIL-induced apoptosis without altering the percentage of cells ultimately killed (82, 83), the long term effects of this combination were examined using colony forming assays (83), an approach that has been utilized previously to examine effects of drugs and cytokines in hematopoietic cells (57, 84). Results of this analysis (Fig. 8G) indicated that a 24-h exposure to TRAIL had little effect on K562 cells, but the combination markedly inhibited colony formation.9 Analysis by the median effect method (Fig. 8H) again demonstrated that the

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8 X. W. Meng, H. Dai, and S. H. Kaufmann, unpublished observations.
9 Jurkat cells could not be utilized for these experiments because they fail to form colonies in soft agar.
effects of the two agents were synergistic, with combination indices of $0.6 \pm 0.04$ and $0.4 \pm 0.2$ ($n = 3$) at the IC$_{50}$ and IC$_{90}$ of the combination, respectively. Collectively, these results indicate that sorafenib treatment, like Mcl-1 down-regulation (Fig. 5), enhances TRAIL-induced killing in K562 cells.

Additional experiments were performed to rule out the possibility that the effects of sorafenib on TRAIL-induced killing were unique to Jurkat and K562 cells. Synergistic induction of apoptosis was also observed in the KBM5 chronic myelogenous leukemia and HL-60 acute leukemia cell lines using several different assays (supplemental Fig. S2). Likewise, T98G human glioblastoma cells exhibited synergistic induction of apoptosis (supplemental Fig. S3A) and inhibition of colony formation (supplemental Fig. S3B) when treated with sorafenib and agonistic anti-DR5 antibodies. Thus, sorafenib appears to enhance TRAIL receptor-mediated killing in a variety of neoplastic cell lines.

**Effect of the Sorafenib/TRAIL Combination in Clinical AML Samples**—Because of current interest in both TRAIL and sorafenib as agents to treat hematological malignancies (see “Discussion”), a final series of experiments was performed to determine whether pharmacological Mcl-1 down-regulation would enhance TRAIL sensitivity in clinical AML specimens. As indicated in Fig. 9A, sorafenib caused Mcl-1 down-regulation in these specimens in a dose-dependent manner. As illustrated in Fig. 9B for one AML isolate, treatment with TRAIL resulted in a limited increase in the number of cells with fragmented DNA. Sorafenib at 5\(\mu\)M likewise had a limited effect by itself (Fig. 9C) but nonetheless enhanced TRAIL-induced apoptosis (Fig. 9B). These proapoptotic effects of the combination were synergistic, as indicated by a combination index of $<1$ (Fig. 9D, open circles). Similar analysis in six additional AML specimens likewise indicated synergy of
the sorafenib/TRAIL combination (Fig. 9D, closed circles and other symbols) in the vast majority of TRAIL exposures.

**DISCUSSION**

Results of this study demonstrate that TRAIL treatment is associated with increased binding of BH3-only family member tBid to Bcl-xL, as well as trafficking of the proapoptotic Bcl-2 family members Bak, Bim, and PUMA to Mcl-1. All of these changes are inhibited by the caspase 8 inhibitor IETD-fmk, suggesting that they occur downstream of DISC formation. The potential importance of the Bak/Mcl-1 interaction is highlighted by the observation that the kinase inhibitor SP600125, which diminishes the binding of Bak to Mcl-1, enhances Bak activation and apoptosis. Moreover, down-regulation of Mcl-1 by shRNA or pharmacologically enhances TRAIL-induced Bak activation and apoptosis in a variety of experimental models. These observations not only provide an explanation for the ability of Mcl-1 to modulate TRAIL-induced apoptosis, but also have potentially important implications for the future development of Mcl-1 modulators and TRAIL receptor agonists.

Initial pulldown assays demonstrated increased binding of Bak to Mcl-1 in TRAIL-treated cells (Figs. 1 and 2). This translocation of Bak to Mcl-1 was not only inhibited by IETD-fmk but also by SP600125, suggesting that it involves caspase 8-mediated JNK activation. If this binding was diminished, e.g. through SP600125 treatment, cells were sensitized to the proapoptotic effects of TRAIL (Figs. 2 and 3), suggesting that the binding between Bak and Mcl-1 is important for neutralizing Bak. To assess this possibility further, Bak activation was analyzed using two different approaches. Although increased binding of an antibody that recognizes the active conformation of Bak was observed after treatment with SP600125 and TRAIL, the change was only a 1.9–2.3-fold increase in mean fluorescence intensity relative to healthy, untreated cells (Fig. 2E and 3D). Because this change seemed small relative to the increase in apoptotic cells (e.g. Fig. 3B), Bak activation was also examined by cross-linking Bak oligomers in isolated mitochondria. We observed that optimal cross-linking required BMH concentrations ~80-fold lower than reported in the original description of this method (Fig. 3E). Nonetheless, when optimal conditions were utilized, SP600125 was found to markedly enhance TRAIL-induced Bak oligomerization (Fig. 3F). The cause of the SP600125-induced increase in Bak oligomerization requires further investigation. Although recent studies have identified a JNK phosphorylation site on Mcl-1 (85), mutation of this site did not affect TRAIL-induced Bak binding, suggesting that JNK is likely acting through another phosphorylation site.

Even though TRAIL treatment results in increased binding of Bak to Mcl-1, Bak binding does not increase (Fig. 1B). Additional experiments have demonstrated increased binding of Bak to Mcl-1 in etoposide-treated K562 cells. Thus, the failure to detect Bak in the Mcl-1 pulldowns does not reflect interference of the S peptide tag with formation of Bak-Mcl-1 complexes. Instead, our results suggest that Bak plays a predominant role during TRAIL-induced apoptosis in K562 cells.

Mcl-1 also bound increasing amounts of Bim and PUMA during TRAIL-induced apoptosis (Fig. 4A). In contrast to the Bak/Mcl-1 interaction, binding of Bim and PUMA was diminished after treatment with IETD-fmk but not SP600125. Further studies are required to identify the signal transduction pathways that result in displacement of Bim and PUMA from their endogenous binding partners. Nonetheless, these observations suggest that TRAIL treatment is accompanied by extensive trafficking of proapoptotic Bcl-2 family members that has previously been largely unrecognized.
FIGURE 8. Induction of apoptosis by the TRAIL/sorafenib combination in K562 cells. A–E, synergistic induction of apoptosis. Cells treated for 24 h with diluent, 25 ng/ml TRAIL, 5 μM sorafenib, or 25 ng/ml TRAIL + 5 μM sorafenib (combination) were permeabilized with Triton X-100, stained with propidium iodide, and subjected to flow microfluorimetry to assess the percentage of particles with 2n DNA content (A) or fixed in 3:1 methanol/acetic acid, stained with Hoechst 33258, and examined by fluorescence microscopy for apoptotic nuclear changes (C, arrowhead). B and D summarize results of samples shown in A or C as well as additional treatments in the same experiments. E shows combination index values for data shown in D. Solid lines, second-order regression lines. F, after K562 cells treated for 24 h with diluent (lane 1), 10 μM sorafenib (lane 2), 12.5 ng/ml TRAIL (lane 3), or 10 μM sorafenib + 12.5 ng/ml TRAIL in the absence (lane 4) or presence (lane 5) of 50 μM Z-VAD(OMe)-fmk, whole cell lysates were subjected to SDS-PAGE followed by blotting for the indicated antigen. β-Actin served as a loading control. Arrows, known caspase cleavage products (15). PARP, poly(ADP-ribose) polymerase. G, K562 cells were treated for 24 h with the indicated concentrations of TRAIL and sorafenib, washed, and plated in 0.3% agar. After 14 days, colonies were counted and expressed relative to diluent-treated samples. Error bars, mean ± S.D. from quadruplicate samples. H, combination index values calculated from the data in G.
In contrast to Bim and PUMA, tBid is not detectably associated with Mcl-1 after TRAIL treatment (Fig. 4D). These observations appear to be at odds with a recent report that tBid is able to bind Mcl-1 in a yeast two-hybrid system (32). Although it is possible that our results reflect an effect of the S peptide tag on Mcl-1 or the FLAG tag on Bid, the ability to pull tBid down with S peptide-tagged Bcl-xL argues against this explanation. Instead, our results are entirely consistent with previous reports showing that Mcl-1 has a $>$10 $\mu$M $K_d$ for tBid (33, 34).

Collectively, the results in Figs. 1, 2, and 4 suggest that TRAIL induces multiple changes in the interactions between pro- and antiapoptotic Bcl-2 family members. Although previous studies have focused extensively on tBid as an agent of mitochondrial disruption in type II cells (3, 30, 31), a variety of earlier observations suggests that tBid provides only part of the mitochondrial signal. In particular, TRAIL has been observed to induce cytochrome $c$ release and caspase 9 activation in Bid $^{-/-}$ mouse embryo fibroblasts (86). More recently, Bim siRNA has been reported to inhibit TRAIL-induced apoptosis in hepatocytes (29). Our results extend these findings by showing that, in addition to Bak (Fig. 1 and Ref. 87), Puma and Bim are constitutively bound to Mcl-1 (Figs. 1B and 4A). Moreover, we demonstrate that TRAIL treatment is accompanied by changes in binding of multiple partners to Mcl-1 (Figs. 1B and 4A) and that Mcl-1 down-regulation enhances TRAIL-induced apoptosis (Fig. 5). These observations are consistent with recent evidence that Bcl-2 family members participate in a complex network in which proapoptotic BH3-only polypeptides induce apoptosis by binding and neutralizing antiapoptotic Bcl-2 homologs (88, 89). Our observations suggest the model shown in Fig. 10, which focuses for simplicity on Mcl-1 as a major component in this dynamic web of protein/protein interactions. Importantly, this model accounts not only for the observations in this paper, but also for the ability of Mcl-1 overexpression or Bim siRNA to inhibit TRAIL-induced apoptosis (29), all of which are difficult to explain based on the role of tBid alone.

In view of the observation that Mcl-1 binds a variety of proapoptotic Bcl-2 family members, we examined the effect of Mcl-1 down-regulation on TRAIL sensitivity. As indicated in

**FIGURE 9.** Effect of sorafenib on TRAIL-induced apoptosis in human AML specimens. A, two different AML samples were treated for 24 h with the indicated concentration of sorafenib in vitro. At the completion of the incubation, whole cell lysates were prepared and subjected to SDS-PAGE followed by blotting for Mcl-1 or, as a loading control, $\beta$-actin. A and C, marrow mononuclear cells from one AML patient treated for 24 h with the indicated concentrations of TRAIL in the absence or presence of 5 $\mu$M sorafenib (B) or the indicated concentrations of sorafenib alone (C) were fixed, stained with propidium iodide, and subjected to flow microfluorimetry. Shown is the percentage of cells with $<$2n DNA content determined as illustrated in Fig. 2F and 8A. D, combination index values from sample shown in B and C (open circles) and six additional AML samples (each symbol type represents a different clinical sample). The amount of spontaneous apoptosis in these AML samples under the conditions of the assay varied between 10 and 25% as reported previously (60).

**FIGURE 10.** Proposed model of Bcl-2 family interactions affected by TRAIL. As described in the text, TRAIL treatment results in Bak activation and apoptotic changes that are modulated by a variety of Bcl-2 family interactions. Constitutive but dynamic interactions between Bak, Bim, Puma, and Mcl-1 are demonstrated in Figs. 1B and 4A. Interactions between tBid and Bcl-xL but not Mcl-1 are depicted in Fig. 4D and F. Effects of SP600125 on Bak activation and apoptosis are shown in Fig. 2D–F, and Fig. 3C, D, and F. Effects of Mcl-1 down-regulation and Bim shRNA on Bak activation and/or apoptosis are shown in Figs. 5–8 and supplemental Figs. S2 and S3. The effect of Bim siRNA was recently reported by Han et al. (29).
Bcl-2 Family Trafficking during TRAIL-induced Apoptosis

Fig. 5, shRNA-mediated Mcl-1 down-regulation resulted in enhanced TRAIL-induced apoptosis. This effect appeared to be greater than that of SP600125 treatment (cf. Figs. 2G and 5B), likely reflecting the fact that Mcl-1 down-regulation affects the action of multiple proapoptotic Bcl-2 family members, whereas SP600125 affects only Bak binding to Mcl-1. Additional experiments indicated that the effect of Mcl-1 down-regulation can be reversed by overexpression of shRNA-resistant Mcl-1 (Fig. 5C) or by overexpression of Bcl-2,4 which would also be expected to bind the proapoptotic Bcl-2 family members ordinarily bound by Mcl-1.

A final series of experiments examined the effect of pharmacological Mcl-1 down-regulation. Based on the recently reported ability of sorafenib to down-regulate Mcl-1 (49, 50), we examined the effects of combining sorafenib with TRAIL or agonistic anti-TRAIL receptor antibodies. Results of this analysis demonstrated that sorafenib simultaneously down-regulated Mcl-1 and sensitized multiple cell lines to TRAIL. This ability of sorafenib to enhance TRAIL sensitivity was demonstrated using assays for a variety of features of the apoptotic phenotype, including DNA fragmentation, changes in nuclear morphology, caspase-mediated cleavage of intracellular substrates, and externalization of phosphatidylserine (Figs. 6–8),4 Moreover, sorafenib diminished long term survival of TRAIL-treated cells as indicated by diminished colony formation after treatment with the combination (Fig. 8, G and H). Examination of a wide variety of other factors that have been reported to affect TRAIL sensitivity, including levels of c-FLIP, XIAP, and of a wide variety of other factors that have been reported to affect TRAIL sensitivity, including levels of c-FLIP, XIAP, and Bcl-xL (supplemental Fig. S1), failed to demonstrate any effect of sorafenib on levels of these other polypeptides in K562 cells. Similar sensitization was observed when cells were treated with agonistic anti-TRAIL receptor antibodies (Fig. 7A and supplemental Fig. S3) or agonistic anti-Fas antibodies (Fig. 7C). Moreover, sorafenib also sensitized clinical AML specimens to TRAIL (Fig. 9).

This ability of sorafenib to enhance death ligand activity has potential practical applications. TRAIL and agonistic TRAIL receptor antibodies are currently undergoing extensive clinical testing in hematological malignancies as well as solid tumors (1–3). Sorafenib, an inhibitor of multiple serine/threonine kinases, is approved for the treatment of renal cell carcinoma and is also currently undergoing phase II testing in other solid tumors and hematological malignancies (90, 91). It is important to emphasize that enhancement of TRAIL sensitivity was evident at sorafenib concentrations of 2.5–10 \( \mu \text{M} \), concentrations that are readily sustainable in the clinical setting (92, 93). Moreover, TRAIL sensitivity was enhanced even at TRAIL concentrations below 1 ng/ml (Fig. 6, A and B), indicating that the sensitization does not necessarily require high concentrations of TRAIL. Thus, further preclinical and possible clinical study of the sorafenib/TRAIL combination appears warranted.

While this manuscript was being revised, we became aware of two additional studies demonstrating the ability of sorafenib to enhance TRAIL sensitivity (94, 95). The present results extend these studies by providing new insight into the mechanism by which Mcl-1 antagonizes TRAIL, by showing that sorafenib also sensitizes cell lines to agonistic anti-DR5 and anti-Fas antibodies, and by demonstrating synergy of the TRAIL/sorafenib combination in TRAIL-resistant clinical AML specimens.

In summary, results of this study indicate that multiple proapoptotic Bcl-2 family members, including Bak, Bim, and PUMA, traffic to Mcl-1 during TRAIL-induced apoptosis. When Mcl-1 levels are insufficient to neutralize these polypeptides, Bak activation and subsequent cell death are increased. Accordingly, further study of Mcl-1 modulation as a potential strategy for enhancing the sometimes limited proapoptotic effects of TRAIL receptor agonists appears to be warranted.

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