Supplementary Methods

PCR Analysis

PCR was performed on genomic DNA extracted from the following six cell lines: KG-1a, HL-60, Kasumi 1, U937, Kasumi 3, and K562. Specifically, the analyzed regions were the 6 exonic regions of *BID*. Genomic DNA was extracted from the frozen cell pellets by incubating the pellets in DNA extraction buffer (50 mM Tris (pH 8), 100 mM EDTA (ethylenediaminetetracetic acid), 100 mM NaCl, 1% SDS) and proteinase K (Qiagen, Valencia, CA) overnight at 56^{o}C. Lysates were then treated with RNase A (Qiagen, Valencia, CA) for 1 h at 37^{o}C, phenol:chloroform extracted, and DNA was precipitated using isopropanol. Precipitated DNA was resuspended in Tris (10 mM)-EDTA (1 mM) and the DNA was quantified using the Quant-iT (Invitrogen, Carlsbad, CA) Picogreen ds DNA assay kit according to the manufacturer's instructions (Invitrogen #Q-33120). PCR primers were designed to amplify the entire exons and incorporated M13 tags for sequencing. The resulting PCR products (5 µl) were incubated with 2 µl ExoSAP-IT (Upstate Biosciences #78200) for 15 minutes at 37°C to digest any single stranded DNA, followed by 15 minutes at 80°C to inactivate the ExoSAP. The resulting double stranded DNA was then subject to Sanger sequencing using primers for the M13 tags.

Cell Culture

The cells of each leukemic cell lines were lysed using 100 µl RIPA (radioimmunoprecipitation assay) protein lysis buffer supplemented with protease and phosphatase inhibitors (Sigma phosphatase inhibitors 2 and 3; #P5726, #P0044): 50 mM Tris-CL, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Triton X-100, 0.1% SDS. Each sample was briefly vortexed and then incubated on ice for 30 minutes; the time on ice allowed for full cell lysis as well as prevention of protein degradation. The samples were then spun at 12,000 revolutions per minute (rpm) at 4°C for 15 minutes. The supernatant (soluble protein fraction) was transferred to a new microcentrifuge tube for protein quantification and the pellet was stored at -80°C.

Western Blotting: Sample Preparation

NuPAGE LDS, to achieve a total concentration of 40 µg protein per 21 µL total sample volume. Several concentrations of NuPAGE LDS (lithium dodecyl sulfate) Sample Buffer with 50 mM DTT (dithiothreitol) were prepared; the use of more than one concentration was to allow for full protein denaturing, but also within the parameters of the required sample volume as dictated by gel well capacity. DTT, a strong reducing agent, was added to the buffer to further increase the buffer's protein denaturing capabilities.

Westerns were run with variations in protein concentrations to find the best amount of protein to enable detection of Bid. Each sample was diluted, vortexed for two seconds to mix the sample, quick spun (microcentrifuge max speed achieved and held for two seconds), incubated at 95°C for ten minutes to further ensure complete protein denaturation, and then quick spun once more to ensure the sample was at the bottom of the tube for pipetting ease.

Protein Quantification

Samples are prepared with a solution of copper ions, which then complex with the protein; folin phenol (phospho-molybdic-phosphotungstic reagent) is then added, which reacts with the protein-copper complex, inducing a color change to blue. Therefore, the greater a sample's protein concentration, the darker the sample will be, and the greater the opaqueness level the spectrophotometer will gauge; numeric values are provided that allow for precise determination of protein concentration.

Samples were prepared by diluting 2 µL lysate with 23 µL ultrapure (milli-q) H_2O. Required reagents in the kit (copper ion solution and folin phenol) were prepared and added to each sample; 200 µL of each sample was added to a 96-well microtiter plate, which was then read by a spectrophotometer at 750 nm. Given the resulting protein concentrations, each sample was diluted sufficiently to fit within the range of the BSA standard if necessary. In cases where dilution was necessary, the assay was run a second time in order to have all protein concentrations fit within the BSA standard.

Western Blotting: Loading and Running the Gel

Novex Pre-Stained Protein Standard was used as the molecular weight marker (#LC5800). Invitrogen NuPAGE 1x MOPS (3-(N-morpholino)propanesulfonic acid) SDS Running Buffer (Invitrogen, catalog # NP0001,

lot # 860421) was prepared from a 20x stock. Gels were run according to NuPAGE protocol at approximately 200 millivolts (mV) for 50 minutes.

Western Blotting: Antibody Treatment of Membranes

In addition to the rabbit-produced total Bid antibody (tBid), antibodies used in this project were specific to the known phosphorylation sites: rabbit-produced phospho Bid (pSer-65) and rabbit-produced phospho Bid (pSer-78); horseradish-peroxidase conjugated goat-produced α-rabbit antibodies were used as secondary antibodies for all of the Bid specific antibodies. α-β-Actin was used as a loading control, with horseradish-peroxidase conjugated goat-α-mouse as the secondary antibody. Primary antibodies were obtained from several sources: tBid: Santa Cruz Biotechnology ((FL-195): sc-11423, raised against amino acids 1-195), phosphoSer65: Abcam (ab59975), phosphoSer78: Bethyl Laboratories (A300-528A), Actin: Sigma-Aldrich (clone AC-15, #A1978).

Membranes were blocked in 10% nonfat dry milk in TBST (Tris buffered saline with Tween-20; 250 mM Tris, 1.5 M NaCl, 20 mM KCl, pH 7.4, 0.1% Tween-20) for 1 hour at 4°C on a rocking platform.

Membranes were then incubated in primary antibody diluted in TBST plus 1% milk (rabbit pSer-65 & pSer-78 1:10,000; Actin 1:1,000) for 1 hour on 4°C shaker or overnight for phosphorylated Bid; washed for 20 minutes in TBST buffer, and incubated in secondary antibodies diluted in TBST plus 1% milk (1:10,000) for 1 hour at 25°C. Membranes were washed for one hour in TBST to remove any excess antibody.

Following membrane incubation, blots were treated with the ECL kit reagents: peroxidase, hydrogen peroxide, and cyclic diacyl hydrazides, such as luminol (Kricka, 2, 3, 1987). Peroxidase catalyzes the reaction between cyclic diacyl hydrazides and hydrogen peroxide, yielding light (Kricka, 2, 3, 1987). These chemicals enhance the chemiluminescence induced by horseradish peroxidase (HRP), which is where the antibodies have affixed to the protein in question (Kricka, 2, 3, 1987).